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(54) Title: A METHOD OF MONITORING PANCREATIC TISSUE VIABILITY

(57) **Abstract**

The present invention relates generally to a method of monitoring the viability of pancreatic tissue and, more particularly, to a method of monitoring the viability of transplanted pancreatic tissue. The method of the present invention is useful, *inter alia*, for monitoring the viability of insulin producing fetal pancreatic tissue which has been transplanted into diabetic subjects.

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A METHOD OF MONITORING PANCREATIC TISSUE VIABILITY

FIELD OF THE INVENTION

The present invention relates generally to a method of monitoring the viability of pancreatic tissue and, more particularly, to a method of monitoring the viability of transplanted pancreatic tissue. The method of the present invention is useful, *inter alia*, for monitoring the viability of insulin producing fetal pancreatic tissue which has been transplanted into diabetic subjects.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Fetal porcine insulin producing cells are capable of normalising blood glucose levels when transplanted into diabetic rodents (1-4). However, fetal β cells (the pancreatic insulin producing cells) are characterised by their inability to secrete insulin when exposed to glucose (5, 6, 7). With time, fetal β cells do develop an ability to secrete insulin when exposed to glucose, probably because oxidative metabolism of glucose improves and ability to generate adenosine triphosphate, which is required for signal transduction of the final insulin secretory pathway, increases (8, 9). The time taken to achieve this goal is 2 to 4 months during which time there is an increase in mass of β cells (2). It is the inability of fetal β cells to respond in an adult manner to glucose that is responsible for their failure to function in the immediate post-transplant period. Insulin is not secreted during this period and blood glucose levels of diabetic recipients are not lowered. Several months after fetal pancreatic tissue is transplanted it will mature (2, 7), insulin will be secreted and blood glucose levels lowered (1-4).

During this period of development it is only possible to determine whether a graft has remained viable or has been rejected, either because of primary non-function or cellular rejection, by preparing a graft biopsy to histologically examine the fetal graft. This is necessarily an invasive procedure which is difficult to perform repetitively on a routine basis. Measuring levels of insulin or C-peptide in the immediate post-transplant period is of no value since these hormones are not released in levels measurable in a radioimmunoassay until several months after transplantation. Accordingly, there is need to develop a non-invasive method of monitoring the viability of pancreatic transplant tissue.

In work leading up to the present invention the inventors have unexpectedly found that the level of molecules which are produced by transplanted pancreatic tissue during the post-transplant period, but prior to the initiation of insulin production, are indicative of the viability of the transplanted tissue.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Sequence Identity Numbers (<400> x) for the amino acid sequences referred to in the specification are defined following the bibliography.

One aspect of the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of a pancreatic tissue derived molecule, or derivative thereof, in the body fluid of said mammal.

Another aspect of the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of a transplant tissue derived molecule, or derivative thereof, in the body fluid of said mammal which molecule is produced prior to the initiation of insulin production by said transplanted pancreatic tissue.

Still another aspect of the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the body fluid of said mammal.

Yet another aspect of the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of transplant derived amylin, or derivative thereof, in the body fluid of said mammal.

Still yet another aspect of the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the plasma of said mammal.

A further aspect of the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant derived amylin, or derivative thereof, in the plasma of said mammal.

Another further aspect of the present invention relates to a method of monitoring transplanted fetal ICC viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the plasma of said mammal.

Yet another further aspect of the present invention relates to a method of monitoring transplanted fetal ICC viability in a mammal said method comprising screening for the modulation of the level of transplant derived amylin levels, or derivative thereof, in the plasma of said mammal.

Still yet another further aspect of the present invention provides a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived mature pancreatic polypeptide levels in the plasma of said mammal.

In another aspect there is provided the method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived icosapeptide levels in the plasma of said mammals.

In yet another aspect the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for an increase in the level of a transplant tissue derived molecule or derivative thereof in the plasma of said mammal.

In still yet another aspect there is provided a method of monitoring for an improvement in transplanted pancreatic tissue viability in a mammal said method comprising screening for an increase in the level of a transplant tissue derived molecule, or derivative thereof, in the plasma of said mammal.

In a further aspect there is provided a method of monitoring for a decrease in transplanted pancreatic tissue viability said method comprising screening for a decrease in the level of a transplant tissue derived molecule, or derivative thereof, in the plasma of said mammal.

Another aspect of the present invention provides a diagnostic kit for assaying body fluid samples comprising in compartmental form a first compartment adapted to contain an

agent for detecting a pancreatic tissue derived molecule and a second compartment adapted to contain reagents useful for facilitating the detection by the agent in the first compartment. Further compartments may also be included, for example, to receive a biological sample. The agent may be an antibody or other suitable detecting molecule.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a photographic representation of pancreatic polypeptide (A) and β (B) cells in the pancreas of a fetal pig of gestational age 90 days. These endocrine cells are present both as single cells and in clumps, but not as islets. In contrast, these cells are found in islets in the pancreas of an adult pig. Pancreatic polypeptide cells (C) are in the periphery and β cells (D) in the centre of the islet. Immunoperoxidase stain; bar is 75 μm.

Figure 2 is a graphical representation of the secretion of pancreatic polypeptide and insulin from groups of 100 ICCs exposed to either 10 mM carbachol or 1 nM secretin for 2 hours. Values are mean ± SEM. n=30 for each group. *P<0.001.

Figure 3 is a graphical representation of the plasma levels of pancreatic polypeptide in SCID mice 4 days after being transplanted with fetal porcine pancreatic explants. Levels were measured in blood collected after intraperitoneal injection of (A) 4.1 μmol/kg carbachol. and (B) 0.03 μmol/kg secretin. There were four groups of mice (n=4-6) in each series of injections - transplant/stimulus; transplant; stimulus; untreated. Data, mean ± SEM, were analysed by ANOV. In A, P=0.003 **transplant/carbachol group > all other groups; *transplant group, carbachol group > untreated group. In B, P<0.001. *transplant/secretin group, transplant group > secretin group, untreated group.

Figure 4 is a graphical representation of levels of pancreatic polypeptide and porcine insulin in SCID mice transplanted with fetal porcine pancreatic explants compared with time since transplantation. There were six values at each time point. Data are expressed as mean ± SEM. A. Plasma levels of pancreatic polypeptide. ANOV: P<0.001. **Day 4, week 1 > weeks 2-12; *weeks 2,3 > weeks 6-12. B. Levels of pancreatic polypeptide in the grafts (extracts of left and right renal transplants combined). ANOV. P<0.001. **Day 4, weeks 2,3 > weeks 6-12. *week 1 > weeks 6,12. C. Levels of insulin in the grafts (extracts of left and right renal transplants combined). ANOV. P<0.001. **Weeks 6-12 > day 4 - week 2. *week 3 > week 1.

Figure 5 is a photographic representation of pancreatic polypeptide cells in a porcine fetal pancreatic graft 1 week after being transplanted beneath the renal capsule of SCID mouse. The percentage of pancreatic polypeptide cells in this graft was 29%, as compared to 11% for the β cells. Immunoperoxidase stain; bar is 75 μm.

Figure 6 is a graphical representation of levels of pancreatic polypeptide and porcine insulin in BALB/c mice transplanted with fetal porcine pancreatic explants compared with time since transplantation. There were 4-5 values at each time point. Data are expressed as mean ± SEM. A. Plasma levels of pancreatic polypeptide. ANOV: P=0.001. *Day 2>days 4 - 14 and no transplant. B. Levels of pancreatic polypeptide in the grafts (extracts of left and right renal transplants combined). ANOV. P<0.001. **Day 2>days 7,14 *days 4,7>day 14. C. Levels of insulin in the grafts (extracts of left and right renal transplants combined). ANOV. P<0.001. *Days 2,4>days 7,14.

Figure 7 is a schematic representation depicting porcine and human pancreatic polypeptide amino acid sequences [<400>1 and <400>2, respectively]. The amino acids which appear in italics indicate the amino acid sequence differences between the porcine and human pancreatic polypeptide sequences.

Figure 8 is a graphical representation of immune affinity chromatography of pancreatic polypeptide using a pancreatic polypeptide antibody matrix. Human serum spiked with ^{125}I labeled human pancreatic polypeptide standards was mixed with the matrix. Unbound proteins were washed off from the matrix followed by specific elution of matrix bound proteins. The amounts of pancreatic polypeptide present in the fractions were detected by measuring radioactive counts.

Figure 9 is a graphical representation of a mass spectrometry result of a porcine and human pancreatic polypeptide mixture.

Figure 10 is a schematic representation depicting porcine and human icosapeptide amino acid sequences [<400>3 and <400>4, respectively].

Figure 11 is a schematic representation of the porcine and human amylin amino acid sequences [<499>5 and <400>6, respectively].

Figure 12 is a graphical representation of the standard curve for porcine amylin standards using a two-antibody sandwich immune assay. An antibody coated plate was incubated with porcine amylin standard solutions of 100, 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 pg/ml concentrations. Biotin-labeled antibody was next bound to the antigen, followed by incubating with neutravidin and biotin-labeled peroxidase. Colour development was measured at 620 nm, 30 minutes following the addition of the developing solution. The non-specific binding in the assay was 0.423.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the unexpected determination that molecules which are produced by transplanted pancreatic tissue during the post-transplant period, but prior to insulin production, can be utilised as an indicator of transplant viability. Accordingly, the inventors have developed a method of monitoring the viability of pancreatic tissue by screening for the levels of such molecules.

Accordingly, one aspect of the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of a pancreatic tissue derived molecule, or derivative thereof, in the body fluid of said mammal.

More particularly, the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of a transplant tissue derived molecule, or derivative thereof, in the body fluid of said mammal which molecule is produced prior to the initiation of insulin production by said transplanted pancreatic tissue.

Reference to a molecule being produced "prior to the initiation of insulin production" should be understood to mean that production of the molecule of interest by the transplanted tissue occurs prior to the secretion of insulin by the transplanted pancreatic tissue. It should be understood that the production of this molecule may either cease prior to insulin secretion or it may continue being produced following the onset of insulin secretion. Further, the production of the molecule of interest may be either constitutive or the result of stimulation.

Reference to a "transplant tissue derived molecule" should be understood as a reference to any proteinaceous of non-proteinaceous molecule, or derivative thereof, which is produced by one or more cells comprising the donor tissue. Reference to a "pancreatic cell" should be understood as a reference to a cell comprising the transplanted pancreatic tissue.

Preferably, said pancreatic tissue derived molecule is pancreatic polypeptide or amylin.

Accordingly, the present invention preferably relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the body fluid of said mammal.

In another preferred embodiment the present invention relates to a method of monitoring mammalian transplanted pancreatic tissue viability said method comprising screening for the modulation of transplant derived amylin, or derivative thereof, in the body fluid of said mammal.

Reference to "body fluid" should be understood to include reference to fluids derived from the body of said mammal, such as but not limited to, blood (including all blood derived components, for example, serum and plasma), urine, tears, mucus and fluids which have been introduced into the body of said mammal and subsequently removed such as, for example, the saline solution extracted following lavage. Preferably, the body fluid is blood and even more preferably plasma. Reference hereinafter to plasma should be read as including reference to all other body fluids.

According to this preferred embodiment, the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the plasma of said mammal.

In another preferred embodiment, the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant derived amylin, or derivative thereof, in the plasma of said mammal.

"Transplanted" should be understood to refer to the grafting of donor tissue onto or into recipient organs. For example, introducing donor cells beneath the renal capsule of the kidney or into the pancreas. It should also be understood to encompass otherwise introducing donor tissue into a donor recipient, for example, by whole organ transplant. Said transplantation may be "syngeneic", "allogeneic" or "xeneogeneic" with respect to the individuals within an animal species from which the pancreatic tissue is isolated (herein referred to as "donors") and the individuals into which said tissue is introduced (herein referred to as "recipients"). A "syngeneic" process means that the donor has the same MHC genotype as the recipient. A "allogeneic" process is where the donor and recipient are of the same species but are MHC-incompatible. A "xeneogeneic" process is where the donor is an individual of a different species to the recipient. An example of a xenogeneic process would be where porcine derived pancreatic tissue is transplanted into a human.

The term "pancreatic tissue" should be read as including reference to any tissue of pancreatic origin. "Tissue" is used in its broadest sense to refer to any form of cellular material, for example isolated cellular populations such as single cell suspensions or organ/tissue fragments or a morphologically intact specimen of all or part of an organ. "Tissue" should also be understood to include reference to cellular material which has not been freshly harvested, for example, cells or specimens which have been thawed from frozen stocks, cells which have been cultered *in vitro* prior to transplantation (for example islet-like cell clusters) or which have been stimulated or otherwise manipulated either prior to or following transplantation. This includes reference to genetically engineered cells.

That said tissue is of "pancreatic origin" should be understood to indicate that at least some of the cells comprising said tissue either directly or indirectly originated from a pancreas. Cells which are harvested from a pancreas are an example of cells directly originating from a pancreas. Cells which are the result of *in vitro* cell division of a cell suspension, adherent cell monolayer or organ culture which was established using tissue harvested from the pancreas are examples of cells indirectly originating from a

mammalian pancreas. This would include, for example, cells derived from a pancreatic cell line. Cells indirectly originating from a mammalian pancreas should also be understood to include genetically engineered cells. This includes, but is not limited to, a non-pancreatic cell which has had one or more genes, normally expressed by a pancreatic cell, either introduced into or up-regulated in that cell. It also includes cells directly or indirectly originating from the pancreas which have been genetically modified, for example, by the introduction of genetic material or the up or down-regulation of the expression of existing genes.

Preferably the tissue of the present invention is fetal pancreatic tissue and even more preferably fetal islet-like cell clusters (referred to herein as "ICC").

According to this most preferred embodiment, the present invention relates to a method of monitoring transplanted fetal ICC viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived pancreatic polypeptide, or derivative thereof, in the plasma of said mammal.

According to another most preferred embodiment, the present invention relates to a method of monitoring transplanted fetal ICC viability in a mammal said method comprising screening for the modulation of the level of transplant derived amylin levels, or derivative thereof, in the plasma of said mammal.

Still more preferably, said fetal tissue is porcine fetal tissue.

The term "viability" should be understood to encompass both the issue of whether a cell is alive, dying or dead and the issue of whether a cell, if alive, is functionally active.

Reference herein to "pancreatic polypeptide" should be read as including reference to all forms of mammalian pancreatic polypeptide (10, 11) and derivatives or homologues thereof. The structure of pancreatic polypeptide shows variation between species. For

example, human pancreatic polypeptide [<400>2] and porcine pancreatic polypeptide [<400>1] differ by two amino acids. The present invention should be understood to relate to pancreatic polypeptide in all its various forms, and derivatives thereof such as, but not limited to, precursor forms of pancreatic polypeptide, allelic variants of pancreatic polypeptide and pancreatic polypeptide isoforms resulting, for example, from differential splicing of pancreatic polypeptide mRNA. In this regard "derivatives" of pancreatic polypeptide should be understood to include the cleavage product generated from post-translational processing of precursor pancreatic polypeptide. Specifically, precursor pancreatic polypeptide is cleaved and gives rise to mature pancreatic polypeptide and a second peptide termed icosapeptide. <400>3 provides the amino acid sequence of porcine icosapeptide and <400>4 provides the amino acid sequence of human icosapeptide.

Reference herein to "amylin" should be read as including reference to all forms of mammalian amylin and derivatives thereof. The structure of amylin shows variation between species. For example, human amylin [<400>3] and porcine amylin [<400>4] differ in both amino acid sequence and polypeptide length. The present invention should be understood to relate to amylin in all its various forms, and derivatives thereof, such as but not limited to, precursor forms of amylin, allelic variants of amylin and amylin isoforms resulting, for example, from differential splicing of amylin mRNA. In this regard "derivatives" of amylin should be understood to include cleavage products generated from post-translational processing of precursor amylin molecules.

Derivatives include fragments, parts, portions, chemical equivalents, analogues, mutants, homologues or mimetics from natural, synthetic or recombinant sources including fusion proteins. Derivatives may be derived from insertion, deletion or substitution of amino acids. Amino acid insertional derivatives include amino and/or carboxylic terminal fusions as well as intrasequence insertions of single or multiple amino acids. Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the protein although random insertion is also

possible with suitable screening of the resulting product. Deletional variants are characterized by the removal of one or more amino acids from the sequence. Substitutional amino acid variants are those in which at least one residue in the sequence has been removed and a different residue inserted in its place. Additions to amino acid sequences including fusions with other peptides, polypeptides or proteins.

The derivatives include fragments having particular epitopes or parts of the entire transplant tissue derived molecule fused to peptides, polypeptides or other proteinaceous or non-proteinaceous molecules. Analogues contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecules or their analogues. To the extent that it is not specified, reference to "pancreatic polypeptide" includes reference to derivatives thereof, and in particular, icosapeptides. Similarly reference to "amylin" includes reference to derivatives thereof.

One preferred embodiment of the present invention provides a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived mature pancreatic polypeptide levels in the plasma of said mammal.

Preferably said pancreatic tissue is fetal ICC and even more preferably said fetal tissue is porcine fetal tissue.

In another preferred embodiment there is provided the method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of transplant tissue derived icosapeptide levels in the plasma of said mammals.

Preferably said pancreatic tissue is fetal ICC and even more preferably said fetal tissue is porcine fetal tissue.

The method of the present invention is exemplified herein using porcine fetal pancreatic tissue as the donor tissue. Although not intending to limit the invention to anyone theory or mode of action, pancreatic polypeptide is secreted from endocrine cells in the fetal pancreas. Porcine pancreatic polypeptide producing cells are thought to be more mature and more resistant than β cells to destruction during the extensive remodelling of the porcine graft that occurs after it is transplanted and before it becomes fully vascularised. Pancreatic polypeptide is therefore detectable from the immediate post-transplant period. Insulin secretion by the transplanted tissue is not detectable under similar conditions due to β cell immaturity and insulin co-localisation with glucagon and/or somatostatin in the one cell (12). Accordingly, blood insulin levels are only useful as a late indicator of graft viability. By screening for the production of porcine pancreatic polypeptide by the porcine pancreatic polypeptide producing cells comprising the transplanted tissue it is possible to determine the viability of the transplant during the immediate post-transplant period. The porcine pancreatic polypeptide is distinguishable from the pancreatic polypeptide produced by the host pancreas on the basis of, for example, differences in amino acid sequences of the donor derived and host derived pancreatic polypeptide. The presence of porcine derived plasma pancreatic polypeptide is an indicator of transplant viability while the absence of donor derived plasma polypeptide is an indicator of transplant death due for example to host rejection of the transplant. Similarly, increasing levels of pancreatic polypeptide is indicative of increasing viable pancreatic cell number while decreasing pancreatic polypeptide levels is indicative of, for example, degeneration of pancreatic tissue which was previously viable.

The term "modulation" refers to increases or descreases in plasma levels of transplant tissue derived molecules such as pancreatic polypeptide and amylin. The increase or decrease may be analysed relative to previously determined levels of the transplant tissue derived molecule, for example, to monitor the ongoing viability of the transplanted tissue. With respect to monitoring the viability of tissue which has been newly transplanted, the level of transplant tissue derived molecule, *per se*, is relevant since prior to transplantation there would be an absence of said transplant tissue derived molecule. Accordingly, the

detection of any transplant tissue derived molecules in recent transplant recipients reflects an increase in the level of these molecules relative to pre-transplant levels. Preferably said modulation is an increase in transplant tissue derived molecule levels.

Accordingly, in a most preferred embodiment the present invention relates to a method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for an increase in the level of a transplant tissue derived molecule or derivative thereof in the plasma of said mammal.

Preferably, said molecule is pancreatic polypeptide or amylin, and more preferably, said pancreatic polypeptide is mature pancreatic polypeptide or icosapeptide.

Still more preferably said pancreatic tissue is fetal pancreatic tissue and even more preferably fetal ICC.

In a still more preferred embodiment said fetal tissue is porcine fetal tissue.

Although the preferred method is to detect an increase in plasma levels of transplant tissue derived molecule, such as pancreatic polypeptide or amylin, the detection of a decrease in said molecule levels may be desired under certain circumstances. For example, as an indicator of graft damage or deterioration. This would permit the commencement of appropriate interventional immunosuppressive treatment. The method of the present invention therefore has widespread applications, including but not limited to, as a non-invasive clinical monitor of early transplant viability and on-going transplant survival. Accordingly, the term "monitoring" as used herein should be understood to refer to both the administration of isolated or random blood tests or the administration of blood testing programs involving repeated testing of a subject over a period of time. It should also be understood that it may be desirable to continue the monitoring program even after the onset of insulin secretion.

In a most preferred embodiment there is provided a method of monitoring for an improvement in transplanted pancreatic tissue viability in a mammal said method comprising screening for an increase in the level of a transplant tissue derived molecule, or derivative thereof, in the plasma of said mammal.

In yet another most preferred embodiment there is provided a method of monitoring for a decrease in transplanted pancreatic tissue viability said method comprising screening for a decrease in the level of a transplant tissue derived molecule, or derivative thereof, in the plasma of said mammal.

Preferably, said molecule is pancreatic polypeptide or amylin, and more preferably, said pancreatic polypeptide is mature pancreatic polypeptide or icosapeptide.

The method of the present invention extends to diagnosing the degree of transplanted pancreatic tissue viability in a mammal based upon an analysis of quantitated transplant tissue derived molecule levels in the plasma of said mammal.

In a related aspect of the present invention, it may be useful to enhance transplant tissue derived molecule production prior to screening. For example, pancreatic polypeptide is secreted from endocrine cells in the pancreas, appropriately called pancreatic polypeptide cells, in response to a vagal stimulus, such as occurs during eating or in response to hypoglycaemia (11). Since vagal innervation of islet and pancreas transplants is not initially present, the secretion of pancreatic polypeptide from such grafts cannot be enhanced by vagal stimulation (11, 13). Exposure of the grafts to a cholinergic agent which mimics the effect of vagal stimulation, but not secretin which requires vagal tone for its effect (11), should stimulate the graft. Carbachol is an example of an agent which enhances pancreatic polypeptide production.

Screening of pancreatic transplant tissue derived molecules, such as pancreatic polypeptide and amylin, can be achieved via a number of techniques such as functional tests,

enzymatic tests or immunological tests, which distinguish between the recipient derived pancreatic molecule and the transplant derived pancreatic molecule. In one particular method the target pancreatic polypeptides in the plasma sample, such as the icosapeptides, are exposed to a specific antibody which may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound target may be detectable by direct labelling with an antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

By "reporter molecule" as used in the present specification, is meant a molecule which, by its chemical nature, provides an analytically identifiable signal which allows the detection of antigen-bound antibody. Detection may be either qualitative or quantitative. The most commonly used reporter molecules in this type of assay are either enzymes, fluorophores or radionuclide containing molecules (i.e. radioisotopes) and chemiluminescent molecules.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different conjugation techniques exist, which are readily available to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, amongst others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable colour change. Examples of suitable enzymes include alkaline phosphatase and peroxidase. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody hapten complex, allowed to bind, and then the excess reagent is washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated,

usually spectrophotometrically, to give an indication of the amount of hapten which was present in the sample.

Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. As in the EIA, the fluorescent labelled antibody is allowed to bind to the first antibody-hapten complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to the light of the appropriate wavelength the fluorescence observed indicates the presence of the hapten of interest. Immunofluorescence and enzyme immunoassay techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules, may also be employed.

In another method, the target molecule, such as pancreatic polypeptide, is detected by immune affinity chromatography and mass spectrometry. This method is based on the extraction of both non-transplant and transplant derived pancreatic polypeptide (for example, human recipient derived pancreatic polypeptide and porcine donor-derived pancreatic polypeptide) by an antibody which does not discriminate between homologues of pancreatic polypeptide. Extraction can be achieved, for example, utilising the technique of immune affinity chromotography. The isolated pancreatic polypeptide is then analysed by mass spectrometry which can differentiate between homologues of pancreatic polypeptide, such as between porcine and human pancreatic polypeptide.

Another aspect of the present invention provides a diagnostic kit for assaying body fluid samples comprising in compartmental form a first compartment adapted to contain an agent for detecting a pancreatic tissue derived molecule and a second compartment adapted to contain reagents useful for facilitating the detection by the agent in the first

compartment. Further compartments may also be included, for example, to receive a biological sample. The agent may be an antibody or other suitable detecting molecule.

Preferably said pancreatic tissue derived molecule is a transplant derived molecule, and even more preferably, pancreatic polypeptide or amylin.

Preferably said body fluid is plasma.

The present invention is exemplified herein with respect to humans receiving porcine derived pancreatic transplants. However this is done with the understanding that the present invention extends to any mammal being the donor or recipient including humans, livestock animals (e.g. sheep, cows, horses, donkey) laboratory test animals (e.g. rats, guinea pigs, rabbits, hampsters) companion animals (e.g. dogs, cats) and captive wild animals (e.g. emus, kangaroos, deer, foxes).

Further features of the present invention are more fully described in the following non-limiting Figures and/or Examples.

EXAMPLE 1

SOURCE OF FETAL PIG TISSUE

Fetal pigs of median gestational age 83 days (range 70-105) were obtained from 11 pregnant sows killed at Bunge Meat Industries (Corowa, New South Wales, Australia), there being 6-15 fetuses (median 12) per litter. The fetuses were air freighted on ice to reach Sydney within 4 hours of the death of the mother, where the pancreases were removed under sterile conditions. The organs were minced into fine fragments of size approximately 1 mm^3 before being transplanted into mice or digested with collagenase to produce islet-like cell clusters (ICCs).

EXAMPLE 2

FORMATION OF ICCS

ICCs were created by partial collagenase digestion of explants with 3 mg/mL collagenase P (Boehringer Mannheim, Mannheim, Germany) in 20 mL Dulbecco's phosphate buffered saline for 15-20 minutes. The digest was washed twice with collagenase buffer, and then cultured in 5% CO_2/air at 37°C in 90 mm petri dishes with RPMI 1640 medium containing 11.2 mM glucose (Trace Biosciences, Castle Hill, Sydney, Australia) and supplemented with 10% fetal calf serum (Trace Biosciences), 20 mM HERPES (Sigma, St. Louis, Missouri) and antibiotics (14). The clusters of cells rounded up in culture over three days. On the third day they were analysed for their endocrine content or stimulated with either secretin or carbachol.

EXAMPLE 3

STATIC STIMULATION *IN VITRO*

ICCs were removed on the third day of culture and placed in groups of 100 in eppendorff tubes. They were washed twice with Hepes buffered Earle's medium, which consists of 124 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 1 mM NaH_2PO_4, 14.3

mM $NaHCO_3$, and 2 mM glucose supplemented with 0.2% bovine serum albumin (Sigma) and 10 mM HEPES. The ICCs were then washed with medium containing the stimuli 10 mM carbamylcholine chloride [carbachol] (Sigma) or 1 nM porcine secretin (Sigma) before being exposed to these stimuli for 2 hours. The tubes were then centrifuged and the supernatant collected for measurement of pancreatic polypeptide and insulin. The hormonal content of the ICCs in the pellet was also measured.

EXAMPLE 4

MEASUREMENT OF PANCREATIC POLYPEPTIDE & INSULIN

Pancreatic polypeptide and insulin were extracted from explants, ICCs and grafts by homogenization of the tissue in acid-ethanol and overnight incubation at 4°C. Levels of porcine pancreatic polypeptide in the extracts, conditioned culture medium and plasma of grafted mice were measured by radioimmunoassay with a kit using a human pancreatic polypeptide standard purchased from Phoenix Pharmaceuticals Inc (Mountain View, California, USA). The cross reactivity of pig and mouse pancreatic polypeptide with the antibody in the kit was 65% and 14% respectively. Pig pancreatic polypeptide is different from human pancreatic polypeptide by two amino acids (position 11: aspartate v. asparagine; position 23: glutamic acid v. aspartic acid); mouse is different from human by six amino acids (position 6: methionine v. valine; position 11: tyrosine v. asparagine; position 21: glutamic acid v. alanine; position 22: threonine v. alanine; position 23: glutamine v. aspartic acid; position 30: threonine v. methionine). Levels of porcine insulin in tissue extracts and conditioned culture medium were measured by an in-house radioimmunoassay using human insulin standards (gift from Novo Nordisk, USA). Attempts to measure levels of porcine insulin in transplanted mice were made using a radioimmunoassay kit for porcine C-peptide (Linco Research St. Charles, Missouri), which is secreted on an equimolar ratio with insulin. Cross reactivity with mouse C-peptide is reported to be $<0.1\%$. The insulin radioimmunoassay was not used because it could not distinguish between porcine and mouse insulin.

EXAMPLE 5

TRANSPLANTATION INTO SCID MICE

Uncultured 1 mm^3 fragments of pig fetal pancreas were transplanted beneath the renal capsule of normoglycaemic male SCID mice as has been described previously (15). In initial experiments the tissue was placed beneath the left renal capsule only, but in later experiments both kidneys were used in order to increase the mass of tissue transplanted. The weight of tissue grafted beneath each renal capsule was 11.8 ± 0.4 mg (mean ± SEM, n=280). Animals were killed by CO_2 gassing at different times after transplantation ranging from 4 days to 12 weeks, with blood collected from the beating heart for estimation of pancreatic polypeptide and insulin.

EXAMPLE 6

HISTOLOGY

Immunohistochemical staining for the pancreatic hormones pancreatic polypeptide, insulin, glucagon and somatostatin was carried out on paraffin sections of fetal and adult pancreas. As well fetal pancreas transplanted for up to 12 weeks beneath the renal capsule of SCID mice was stained for endocrine hormones. The staining technique applied was a sandwich one using primary antibodies to the 4 hormones (Dako Corp., California), complemented by biotinylated second antibodies, streptavidin and the chromogen 3-amino-9-ethylcarbazole (Dako) with eosin as a counterstain. Control staining was conducted omitting the primary antibodies. The percentage of fetal epithelial-like cells staining positively for the various endocrine hormones was estimated in a blinded manner by the one observer with between 1236 and 7626 cells counted for each section analysed.

Haematoxylin and eosin sections of pancreas transplanted into BALB/c mice were made and analysed to document exactly when rejection of the graft commenced.

EXAMPLE 7

STATISTICS

Data were analysed using one way analysis of variance with Duncan's test ($P < 0.05$) applied to separate groups. When variances among the groups were unequal, data were transformed before analysis. The computer statistical package NCSS was used for all calculations (16).

EXAMPLE 8

HISTOLOGY OF FETAL PIG PANCREAS

Cells in fetal pig pancreas containing pancreatic polypeptide were present singly and in clumps (Figure 1A), just as were the other endocrine hormones, insulin, glucagon and somatostatin. This contrasts to their presence in the periphery of islets in adult pig pancreas (Figure 1B). Islets have previously been shown not to be present in the fetal pig pancreas (17).

EXAMPLE 9

PANCREATIC POLYPEPTIDE CONTENT IN EXPLANTS AND ICCS

The levels of pancreatic polypeptide in fetal pig pancreatic explants and ICCs, which are the more purified form in which fetal pig pancreas is often transplanted, are detailed in Table 1. The relative amount of this hormone compared to insulin was higher in ICCs than explants, 5.8 vs 2.7%. The absolute amount of pancreatic polypeptide and insulin in an average graft of explants (11.8 mg) and ICCs (500) was calculated using the data in Table 1. Amounts of pancreatic polypeptide and insulin were higher in ICCs, 7 vs 80 pmol of pancreatic polypeptide and 191 vs 465 pmol for insulin.

Secretion of pancreatic polypeptide from porcine ICCs was stimulated by the parasympathetic agent carbachol but not the gastrointestinal hormone secretin (Figure 2). Insulin secretion was also enhanced by carbachol but not secretin. Content of pancreatic polypeptide and insulin were not significantly different among the groups, the mean levels being 166 and 2006 fmol respectively. The percentage of pancreatic polypeptide content secreted in 2 hours increased from 2.3 ± 0.3% to 4.5 ± 0.4% when ICCs were exposed to carbachol and that of insulin from 1.1 ± 0.1% to 2.3 ± 0.2%.

EXAMPLE 10

OPTIMIZING PLASMA PANCREATIC POLYPEPTIDE LEVELS IN GRAFTED SCID MICE

SCID mice were transplanted with fetal porcine pancreatic explants beneath the capsule of two kidneys since better vascularization of the grafts was achieved in this way. Pancreatic polypeptide could now be readily detected in plasma with levels of 6.2 ± 0.5 pmol/L (n=6) 4 days after transplantation as compared to 4.5 ± 0.3 pmol/L in plasma from untransplanted mice. Attempts were made to increase the secretion of pancreatic polypeptide into blood by injecting the mice with either carbachol or secretin. Only the former stimulus was of benefit, with levels of 11.8 ± 2.0 pmol/L being measured (Figure 3).

EXAMPLE 11

TIME COURSE OF PANCREATIC POLYPEPTIDE LEVELS IN GRAFTED SCID MICE

All of the above plasma pancreatic polypeptide levels were estimated 4 days after transplantation because initial experiments had shown that the pancreatic polypeptide content of the grafts was higher at this time than at 7 and 14 days. A study was then conducted measuring the plasma levels of pancreatic polypeptide as well as the graft content of pancreatic polypeptide at different times for up to 12 weeks after, the time

when the graft should secrete sufficient insulin to normalize blood glucose levels in diabetic mice (2). The results of this study are in Figure 4A. They show that pancreatic polypeptide is secreted from the grafts and can be measured for at least 3 weeks after transplantation. By 6 weeks the levels were no different from those in untransplanted mice. Levels of pancreatic polypeptide were highest for the first week after transplantation (Figure 4A).

Pancreatic polypeptide in the grafts parallelled the plasma pancreatic polypeptide values with levels being higher during the first 3 weeks (Figure 4B). Conversely, insulin levels in the graft were lowest during this period (Figure 4C). The relative amount of pancreatic polypeptide compared to insulin was much higher in the first 3 weeks than that in untransplanted tissue - 59, 87, 74 and 56 at day 4, weeks 1, 2 and 3 post transplant respectively vs. 1.4% before transplantation. The ratios of pancreatic polypeptide to insulin at 6, 8 and 12 weeks post transplant approached pre-transplant levels at 7, 8 and 7% respectively. Plasma levels of porcine C-peptide, a marker of porcine insulin, were unmeasurable at any time even at 6-12 weeks when the insulin content of the graft was highest.

EXAMPLE 12

HISTOLOGY IN GRAFTED SCID MICE

Pancreatic polypeptide cells were readily identified in the grafts (Figure 5). The percentage of pancreatic polypeptide cells was higher during the first 3 weeks after transplantation than at later times (Table 2). In contrast cells containing insulin or glucagon were highest from 3 weeks onwards. The percentage of epithelial cells in the grafts which were positive for any of the three hormones was also greatest from 3 weeks onwards (Table 2).

EXAMPLE 13

PLASMA PANCREATIC POLYPEPTIDE LEVELS DURING REJECTION

If plasma pancreatic polypeptide levels are to be a marker of viability, they should diminish during episodes of rejection. To study this, fetal porcine pancreatic explants were transplanted beneath the renal capsule of immunocompetent mice (BALB/c) and the mice killed 2, 4, 7 and 14 days later.

Histologically cellular rejection was shown to commence at 4 days, with most pancreatic tissue destroyed by 14 days. Pancreatic polypeptide levels were significantly greater than those in ungrafted mice at 2 days after grafting but not thereafter (Figure 6A). Pancreatic polypeptide levels in the graft were highest at 2-4 days (Figure 6B), as were the levels of insulin (Figure 6C), declining as the graft was rejected.

EXAMPLE 14

IMMUNE AFFINITY CHROMATOGRAPHY AND MASS SPECTROMETRY

Both human and porcine pancreatic polypeptide present in the recipient serum are extracted by immune affinity chromatography. Isolated pancreatic polypeptide is then analysed by mass spectrometry, which can differentiate between porcine pancreatic polypeptide and human pancreatic polypeptide.

Immune affinity chromatography is one of the most powerful techniques for the isolation of proteins (18). Under the proper conditions, purification of 1000-10000 fold can be achieved routinely in a single step. Immune affinity chromatography can be divided into three steps:

(1) Preparation of the antibody matrix

(2) Binding the antigen to the antibody matrix

(3) Elution of the antigen

Antigen refers to a protein which specifically binds to the antibody matrix. Matrix is a solid support, for example in the form of a bead.

Methods

(i) Preparation of antibodies for immune affinity chromatography

Polyclonal antibodies against human pancreatic polypeptide were raised in rabbits. Rabbits were immunised by injecting synthetic human pancreatic polypeptide (American Peptide Company) conjugated to keyhole limpet hemocyanin. Intramuscular injections of 50 µg peptide per animal were given as the primary immunisation while subcutaneous injections of 20 µg peptide per animal were given as the secondary immunisation.

Antibody titers for the presence of positive sera were determined by an enzyme immune assay. Microtitre plates were coated by adding 50 µl of antigen (i.e. human pancreatic polypeptide) solution, 2 µg per ml, in coating buffer (0.5M $NaHCO_3$, pH 9.5) and incubated overnight. Coated plates were washed with distilled water, followed by adding blocking buffer (0.1M NaB_3, 0.25% BSA, 0.05% Tween), 50 µl per well. After 1 hour at room temperature, plates were washed with distilled water three times as described. Serum (or purified antibody solution) dilutions of 1:100, 1:1000, 1:10,000 and 1:100,000 (using blocking buffer) were added, 50 µl per well and incubated for 2 hours at room temperature. Plates were washed again followed by incubating with blocking buffer for 10 minutes at room temperature. After washing the plates, peroxidase labeled goat anti-rabbit IgG (KPL), 1 µg/ml, was added, 50 µl per well, for 2 hours at room temperature. After the final washing, TMB Peroxidase Substrate solution (KPL) was added, 100 µl per well, which produces a blue colour. After 5 minutes, 0.18M H_2SO_4 acid solution, 100 µl per well, was added to stop the reaction which produces a dark yellow colour. Absorbance was read by a microplate reader (Labsystems) using a 450 nm filter.

The pH of the serum was adjusted to 8.0 by adding 1/10 volume of 1.0M Tris. The serum (1 ml) was passed through a 1 ml protein A bead column. The beads were washed with 10 ml of 100 mM Tris pH 8.0 and the column eluted with 5 ml of 100 mM glycine (pH 3.0). 1 ml eluted fractions were collected and neutralised by adding 100 µl of 1M Tris (pH 8.0). 20 µl from eluted fraction was used in Bradford protein assay to identify the fractions containing the antibody. Enzyme immune assay was carried out using a sample from this fraction to measure the antibody activity.

This procedure is repeated until the desired amount of antibody is collected. The antibody solution was concentrated by ultrafiltration using Cenriprep filters (Amicon). The concentrated solution was dialysed against PBS to remove Tris and glycine. Protein assay was carried out to quantitate the amount of antibody present.

(ii) Preparation of immune affinity chromatography matrix

For immune affinity chromatography, purified antibodies were covalently bound to a protein A Sepharose matrix. In the methods used by the inventors up to 5mg of purified antibodies were covalently conjugated to 2 ml of protein A sepharose by using dimethyl pimelimidate (DMP).

Diluted 1 ml of antibody solution with 1 ml of borate buffer, pH 8.2. 1 ml of protein A-Sepharose (Pierce) was equilibrated with borate buffer. Antibody solution was mixed with protein A Sepharose for ½ hr at room temperature with gentle shaking, after which the Sepharose beads were washed with excess borate buffer. The Sepharose was washed with 0.2M triethanolamine, pH 8.2, and then resuspended in 1 mg/ml DMP (Pierce) freshly made up in triethanolamine. The mixture was agitated gently at room temperature for 1 hour, and the reaction was stopped by adding 0.1M ethanolamine, pH 8.2. After 10 min, cross-linked beads were washed three times with 0.1M borate buffer supplemented with 0.02% sodium azide.

(iii) Immune affinity chromatography method

In order to establish the immune affinity protocol, human serum containing human pancreatic polypeptide standards were used as samples. Human serum samples spiked with ^{125}I labeled human pancreatic polypeptide standards were used to immune extract pancreatic polypeptide by using the antibody matrix. Serum samples were mixed 1:1 with 0.05M Tris, pH 8.2. The mixture was incubated for ½ hr at room temperature with constant agitation. The beads were removed by gentle centrifugation (500g for 1 minute). The centrifuged beads were washed in sequence with the following three buffers. (1) 0.05M Tris-HCl, pH 8.2, 0.5M NaCl, 1 mM EGTA (2) 0.05M Tris-HCl, pH 8.2, 0.5M NaCl (3) 0.15M NaCl, 0.1M deoxycholate (two washes). Antigens specifically bound to the matrix were eluted from the beads by mixing an equal volume of 0.05M diethylamine, pH 11.5, containing 0.1% deoxycholate and 0.1% SDS for 10 min. This was repeated and the two supernatants were pooled and neutralised by the addition of 0.5M NaH_2PO4. The beads were washed several times in borate buffer and stored in this buffer containing 0.02% sodium azide. The amounts of ^{125}I-pancreatic polypeptide present in the washing and eluted fractions were detected by measuring the radioactive counts using a gamma-counter.

(iv) Processing of immune isolated pancreatic polypeptide for mass spectrometry

Neutralised eluted fractions containing pancreatic polypeptide wer concentrated by ultrafiltration using Centriprep filters (Amicon). This was followed by removal of salts and detergents by dialysis against distilled water. Finally, the samples were used in speed vacuum centrifugation for complete evaporation of liquid, for analysing in mass spectrometry.

(v) Matrix-assisted-laser-desorption-ionization (MALDI)-mass spectrometry of human and porcine standards

Mixtures of porcine and human pancreatic polypeptide standards in distilled water were analysed by MALDI-mass spectrometry. 10 ml of each sample was concentrated by vacuum drying procedure and reconstituted in 2 µl of 0.2% Trifluoroacetic acid. 8 µl aliquots of sinapnic acid (MALDI matrix) were added to each sample, giving total reconstitution volume of 10 µl. 200 pmol of BSA were added to each sample to prevent the loss of proteins due to binding to the surfaces.

These samples were applied directly to the MALDI target in 500 nl aliquots, and analysed in positive ion linear delayed extraction mode, over the 3 to 8 kDa mass range .1:1 mixtures of 100, 10 and 1 pmole human pancreatic polypeptide present in 20 ml of H_2O yielded two separate peaks corresponding to their molecular masses of 4197 and 4182, respectively. Since normal human pancreatic polypeptide level in serum is 50 pmol/l, 20 ml of serum should contain at least 1 pmol of pancreatic polypeptide. Therefore, this lowest detection level is comparable to the normal human pancreatic polypeptide level.

RESULTS

In the first step, purified monoclonal or polyclonal antibodies are covalently attached to a solid phase matrix. There are a large number of different protocols for covalently binding antibodies to a solid phase, but probably the most common is linking antibodies to protein A beads. After the preparation of the antibody-bead matrix, the antigen is bound to the antibodies and contaminating macromolecules are removed by washing. In the third step, the antibody-antigen interaction is broken by treating the immune complex with strong elution conditions, and the antigen is released into the eluate.

(i) Generation of antibody to human pancreatic polypeptide

Rabbit polyclonal antibodies against synthetic human pancreatic polypeptide were developed due to their ability to recognise both human pancreatic polypeptide and porcine pancreatic polypeptide. These antibodies exhibited a high affinity of binding to both human pancreatic polypeptide and porcine pancreatic polypeptide.

An enzyme immune assay was used to detect specific antibodies against human pancreatic polypeptide. In this assay, microtitre plates coated with the antigen (i.e. human pancreatic polypeptide) were incubated with diluted serum solutions. Specific antibodies in the serum would bind to the coated antigen while unbound antibodies and other serum proteins are removed by washing. A labeled second antibody, which binds to the rabbit antibody (e.g. peroxidase conjugated to antibody against rabbit IgG), is then quantitated. This is proportional to the amount of specific antibodies in the test solution (18).

Antibodies present in the rabbit serum were extracted by employing a protein A matrix. Chromatography of serum samples over a protein A bead column is an effective method for purifying antibodies from sera. Antibodies with high affinity binding sites for protein A, such as rabbit antibodies, can be purified conveniently using low salt buffers. The antibodies are eluted by lowering the pH of the buffer.

The purity of the antibody solution was assessed by using an aliquot in a sodium dodecyl sulfate (SDS) polyacrylamide gel. In a pure antibody sample one would observe only the protein bands corresponding to antibody molecules in a stained gel. Techniques that measure the total amount of protein, such as the Bradford assay, were used for protein quantitation of the purified antibody solution. The Bradford method depends on quantitating the binding of a dye, Coomassie Blue, to an unknown protein and comparing the binding to that of different amounts of standard protein, usually bovine serum albumin (19).

(ii) Preparation of antibody matrix

An immune affinity matrix was prepared by binding purified pancreatic polypeptide antibody to protein A Sepharose, followed by cross-linking of the complex with dimethyl pimelimidate (DMP). The antibody molecule is bound to the matrix via a site that is different from the antigen binding site of the antibody. This allows optimal spatial orientation of antibodies, and thus maximum antigen binding efficiency. The affinity matrix was stable to high and low pH buffers without any significant antibody loss.

(iii) Binding pancreatic polypeptide to antibody matrix and elution of pancreatic polypeptide

The pancreatic polypeptide matrix was used in immune affinity chromatography experiments using human serum samples spiked with human pancreatic polypeptide standards. The affinity matrix was used as a batch absorbent to maximise the degree of interaction between antigen and antibody. A protocol was developed for isolating pancreatic polypeptide by using a specific buffer system, which includes a high pH buffer containing detergents for eluting pancreatic polypeptide. By using this protocol, up to 70% of the human pancreatic polypeptide applied to the matrix was isolated in the eluted fraction.

(iv) Mass spectrometry

Mass spectrometry is used in the next part of the experiment, which is able to distinguish proteins that are different from each other by a single amino acid, or more. In this part, isolated pancreatic polypeptide from immune affinity chromatography is analysed for the presence of porcine and human peptides.

A mass spectrometry technique of matrix-asisted-laser-desorption-ionization (MALDI) was employed to detect porcine and human pancreatic polypeptide. In the MALDI technique, the protein sample is mixed in a matrix which absorbs the energy from a laser beam to ionize the molecules. The ionized protein molecules present in the sample produce signals which are identified by their molecular masses. The immune extracted pancreatic polypeptide sample should be extensively dialysed against distilled water to remove salts and detergents, thereby improving the signal produced by the protein of interest.

In the experiments mixtures of porcine and human pancreatic polypeptide standards in distilled water were analysed by MALDI-mass spectrometry. Total pancreatic polypeptide amounts of 100, 10 and 1 pmol gave distinct peaks for porcine and human pancreatic polypeptide, confirming its ability to separate the two peptides. Experiments were also carried out using human serum spiked with porcine and human pancreatic polypeptide standards. Pancreatic polypeptide was isolated from the samples by the immune affinity chromatography protocol.

EXAMPLE 15

MONITORING ICOSAPEPTIDE

The survival and functioning of fetal porcine pancreatic tissue in humans can also be monitored by screening for icosapeptide. Pancreatic polypeptide is synthesised in pancreatic polypeptide cells from a 95 amino acid precursor (20). As a result of post translational processing which occurs in the pancreatic polypeptide cell, pancreatic polypeptide precursor gives rise to pancreatic polypeptide as well as a second peptide called icosapeptide. Pancreatic polypeptide and icosapeptide are secreted in equimolar amounts from the pancreas (11).

Human icosapeptide is 20 amino acids long, and is not as evolutionally well conserved as pancreatic polypeptide. The human sequence when compared with two other known sequences of sheep and dog is different by 6 and 9 amino acids, respectively (21). The

amino acid sequence of the porcine icosapeptide was not known.

Determination of porcine icosapeptide sequence

Reverse transcription using 3'RACE kit (Promega) was carried out. Primers were designed for polymerase chain reactions using the known pancreatic polypeptide DNA sequences of human, mouse, rat, dog, and guinea pig. The target cDNA was amplified by reverse transcription PCR using porcine RNA.

DNA generated from the PCR reactions were cloned, and positive clones were sequenced. DNA sequence of porcine icosapeptide and a part of porcine pancreatic polypeptide were obtained from these experiments.

RESULTS

Experiments were carried out to determine the porcine icosapeptide sequence by using the method of reverse transcription-polymerase chain reaction (RT-PCR). The resultant porcine icosapeptide thus deduced is 19 amino acids long and is only 26% homologous to the human sequence. Therefore, the detection of porcine icosapeptide in the recipient provides another method for monitoring the survival and functioning of the graft.

Polyclonal antibodies are raised in rabbit and sheep against the porcine icosapeptide. Detection of porcine icosapeptide present in the samples can proceed utilising any suitable method known to those skilled in the art, such as enzyme immuno assay and radioimmunoassay.

EXAMPLE 16

MONITORING AMYLIN LEVELS

Amylin is responsible for amyloid formation in humans. Amylin level in human serum is in the range of 10 to 35 pg/ml (22). Porcine amylin is a 32 amino acid peptide, and is only 62% homologous to the human amylin which is a 37 amino acid peptide.

The two-antibody sandwich technique is a useful immunoassay for detecting antigen because of its relatively high sensitivity. This assay is used primarily to determine the antigen concentration in unknown samples. The assay requires two antibodies that bind to non-overlapping epitopes on the antigen. Either two monoclonal antibodies that recognise discrete sites of the antigen, or one batch of affinity-purified polyclonal antibodies can be used (23, 24).

To use the two-antibody sandwich assay using polyclonal antibodies, purified antibody is bound to a solid phase, and the antigen (e.g. amylin in a test solution) is allowed to bind the antibody. Unbound proteins are removed by washing, and the labeled purified antibody is allowed to bind to the antigen. After washing, the assay is quantitated by measuring the amount of labeled antibody that is bound to the antigen-antibody complex. The major advantages of this technique are that the antigen does not need to be purified prior to use and that the assays are very specific (23, 24).

Labeling antibodies by covalent coupling of biotinyl groups is a very common technique used in two-antibody sandwich assays. Biotinylation normally does not have any adverse affect on the antibody, and the coupling conditions are mild. Biotinylated antibodies can be detected by using a bridging molecule such as avidin, strepavidin, or neutravidin together with a biotin-labeled enzyme (e.g. peroxidase). The non-covalent, high affinity interaction between biotin and these molecules allows more signal to be concentrated at the detection site. Neutravidin is a deglycosylated form of avidin, and offers the advantage of a neutral pH to minimise nonspecific absorption. The amount of substrate hydrolysed by

the enzyme is proportional to the amount of antigen in the test solution.

Polyclonal antibodies against porcine amylin raised in rabbits were used for developing a two-antibody sandwich assay. An immune assay was used to detect specific antibodies against porcine amylin. The antibodies exhibiting a high affinity to porcine amylin. The antibodies were extracted from rabbit serum by using a protein A matrix. A batch of purified antibody was reconstituted in a coating buffer, and was used for binding the microtitre plates. A second batch of purified antibody was labeled by biotin to use as the second antibody layer.

The two-antibody sandwich assay was developed to detect porcine amylin in test samples. The signal of the biotin-labeled antibody was amplified by using neutravidin and biotin-labeled peroxidase, and was detected by measuring the colour development. The assay showed a linear range of binding of porcine amylin standards from 30 to 1000 pg/ml. This methodology is utilised to detect the secretion of amylin from fetal porcine islet-like cell clusters.

Methods

(i) Preparation of antibodies

Polyclonal antibodies against porcine amylin were raised in rabbits. Rabbits were immunized by injecting synthetic porcine amylin (Auspep) conjugated to keyhold limpet hemocyanin. Intramuscular injections of 200 ug peptide per animal were given as the primary immunization while subcutaneous injections of 200 ug peptide per animal were given as the secondary immunization.

Antibody titers for the presence of positive sera were determined by an enzyme immune assay. Microtitre plates were coated by adding 50 ul of antigen (i.e. porcine amylin) solution, 10 ug per ml, in coating buffer (0.5M $NaHCO_3$, pH 9.5) and incubated

overnight at 4°C. Coated plates were washed with distilled water, followed by adding blocking buffer (0.1M NaB_3, 0.25% BSA, 0.05% Tween), 50 ul per well. After 1 hour at room temperature, plates were washed with distilled water three times. Serum (or purified antibody solution) dilutions of 1:100, 1:1000, 1:10,000 and 1:100,000 (using blocking buffer) were added, 50 ul per well and incubated for 2 hours at room temperature. Plates were washed again followed by incubating with blocking buffer for 10 minutes at room temperature. After washing the plates, peroxidase labeled goat anti-rabbit IgG (KPL), 1 ug/ml, was added, 50 ul per well, for 2 hours at room temperature. After the final washing, TMB Peroxidase Substrate solution (KPL) was added, 100 ul per well, which produces a blue colour. After 5 minutes, 0.18 M H_2SO_4 acid solution, 100 ul per well, was added to stop the reaction which produces a dark yellow colour. Absorbance was read by a microplate reader (Laysystems) using a 450 nm filter.

The pH of the serum was adjusted to 8.0 by adding 1/10 volume of 1.0M Tris. The serum (1 ml) was passed through a 1 ml protein A bead column. Washing the beads with 10 ml of 100 mM Tris pH 8.0. Eluted the column with 5 ml of 100 mM glycine (pH 3.0). 1 ml eluted fractions were collected and neutralized by adding 100 ul of 1M Tris (pH 8.0). 20 ul from eluted fraction was used in Bradford protein assay to identify the fractions containing the antibody. Enzyme immune assay was carried out, as described above, using a sample from this fraction to measure the antibody activity.

This procedure is repeated until the desired amount of antibody is collected. The antibody solution was concentrated by ultrafiltration using Cenriprep filters (Amicon). The concentrated solution was dialysed against PBS to remove Tris and glycine. Protein assay was carried out to quantitate the amount of antibody present.

(ii) Biotin labelling of antibody

2 mg of IgG was made up in 1 ml of 50mM $NaHCO_3$, pH 8.0, in a test tube. Immediately prior to use, 1 mg of NHS-biotin (Pierce) was dissolved in dimethyl sulfoxide. 75 ul of the dissolved NHS-biotin was added to the test tube containing the IgG and the mixture was incubated for 2 hours at 4°C. The conjugated antibody solution was then dialysed against PBS to remove unreacted biotin.

(iii) Two-antibody sandwich immune assay

Purified (unlabeled) IgG was reconstituted in coating buffer (0.5M $NaHCO_3$, pH 9.5) at a concentration of 10 ug/ml. Microtiter plates (Maxisorp, Nunc) were coated by adding the coating antibody solution (50 ul/well) and incubating overnight at 4°C. The diluting buffer used in the assay was borate-buffered saline (0.17M H_3BO_4, 0.12M NaCl, pH 8.5) containing 0.25% BSA, 0.05% Tween 20, and 0.05% NaN_3. To minimise non-specific binding, the wells were incubated with diluting buffer (100 ul per well) for 1 hour at room temperature, followed by washing twice with distilled water. A standard antigen dilution series was prepared by successive 1:1 dilution of a 1000 pg/ml porcine amylin solution yielding 500, 250, 125, 62.4, 31.25, 15.6, and 7.8 pg/ml solutions in diluting buffer. 50 ul aliquots from each of the antigen standard solution were added to the wells and incubated for 2 to 3 hours at room temperature. The plate was washed 5 times with distilled water. A 10 ug/ml biotin labeled antibody solution was made up in diluting buffer. Labeled antibody was added 50 ul per well and incubated for 1 hour at room temperature, followed by washing 6 times with distilled water. A neutravidin-peroxidase solution was made up by mixing equal volumes of a 10 ug/ml neutravidin (Pierce) solution in diluting buffer (without NaN_3) and a 5 ug/ml biotin-labeled peroxidase (Pierce) solution in diluting buffer (without NaN_3) in a test tube, and was incubated for 1 hour at room temperature. The neutravin-peroxidase solution was added to the plate, 100 ul per well, and incubated for 1 hour at room temperature. After the final washing with distilled water, TMB Peroxidase Substrate solution (KPL) was added, 100 ul per well. Blue colour

development was measured 30 minutes later by a microplate reader (Labsystems) using a 620 nm filter.

The antibody sandwich assay was used to measure amylin secretion from fetal porcine ICC's. Amylin secretion from 200 ICC's was 11.33 ± 3.29 pg per 1 hour, or 32.8 ± 0.9 fmol per hour. Insulin secretion, measured by a radioimmunoassay, was 74.4 ± 15.4 fmol per 1 hour from the same amount of ICC's. Thus, for every mole of insulin secreted, 0.44 moles of amylin are secreted.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

Table 1. Content of pancreatic polypeptide and insulin in fetal pig pancreatic explants and ICCs. Data are mean ± SEM.

Tissue	Pancreatic polypeptide (pmol/mg tissue or ICC)	Insulin (pmol/mg tissue or ICC)	Pancreatic polypeptide/ Insulin(%)
Explants	0.58 ± 0.18	16.2 ± 3.1	2.7 ± 1.7
ICCs	0.16 ± 0.01	0.93 ± 0.19	5.8 ± 1.7

Table 2. Endocrine cells expressed as a percentage of the total number of epithelial cells in untransplanted fetal pig pancreatic tissue and tissue transplanted beneath the renal capsule of SCID mice for varying amounts of time. The column labelled endocrine cells represents the sum of the pancreatic polypeptide, β and α cells.

Time after transplantation (weeks)	Pancreatic polypeptide cells (%)	β cells (%)	α cells (%)	Endocrine cells (%)
untransplanted	8.7	11.5	7.8	28.8
0.5	17.2	13.2	11.6	42.1
1	13.3	15.7	7.7	42.3
3	20.6	30.4	21.5	75.4
9	10.9	27.2	24.3	64.7
12	9.1	34.0	21.5	65.4

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CLAIMS:

1. A method of monitoring transplanted pancreatic tissue viability in a mammal said method comprising screening for the modulation of the level of a transplant tissue derived molecule, or derivative thereof, in the body fluid of said mammal.

2. A method according to claim 1 wherein said molecule is produced prior to the initiation of insulin production.

3. A method according to claim 2 wherein said transplant tissue derived molecule is pancreatic polypeptide.

4. A method according to claim 3 wherein said pancreatic polypeptide is mature pancreatic polypeptide.

5. A method according to claim 3 wherein said pancreatic polypeptide is icosapeptide.

6. A method according to claim 2 wherein said transplant tissue derived molecule is amylin.

7. A method according to any one of claims 2-6 wherein said pancreatic tissue is fetal ICC.

8. A method according to claim 7 wherein said fetal ICC is porcine fetal ICC.

9. A method according to any one of claims 2-8 wherein said modulation is an increase in the level of a transplant tissue derived molecule, or derivative thereof.

10. A method of monitoring for an improvement in transplanted pancreatic tissue viability in a mammal said method comprising screening for an increase in the level of a transplant tissue derived molecule in the body fluid of said mammal.

11. A method of monitoring for a decrease in transplanted pancreatic tissue viability said method comprising screening for a decrease in the level of a transplant tissue derived molecule in the body fluid of said mammal.

12. A method according to claims 10 or 11 wherein said molecule is produced prior to the initiation of insulin production.

13. A method according to claim 10 or 11 wherein said transplant tissue derived molecule is pancreatic polypeptide.

14. A method according to claim 13 wherein said pancreatic polypeptide is mature pancreatic polypeptide.

15. A method according to claim 13 wherein said pancreatic polypeptide is icosapeptide.

16. A method according to claim 10 or 11 wherein said transplant tissue derived molecule is amylin.

17. A method according to any one of claims 10-16 wherein said pancreatic tissue is fetal ICC.

18. A method according to claim 17 wherein said fetal ICC is porcine fetal ICC.

19. A method according to any one of claims 1-18 wherein said body fluid is plasma.

20. A diagnostic kit for assaying mammalian body fluid samples comprising in compartmental form a first compartment adapted to contain an agent for detecting a pancreatic transplant tissue derived molecule and a second compartment adapted to contain reagents useful for facilitating the detection by the agent in the first compartment.

21. A diagnostic kit according to claim 20 wherein said pancreatic transplant tissue derived molecule is pancreatic polypeptide.

22. A diagnostic kit according to claim 208 wherein said pancreatic tissue derived molecule is amylin.

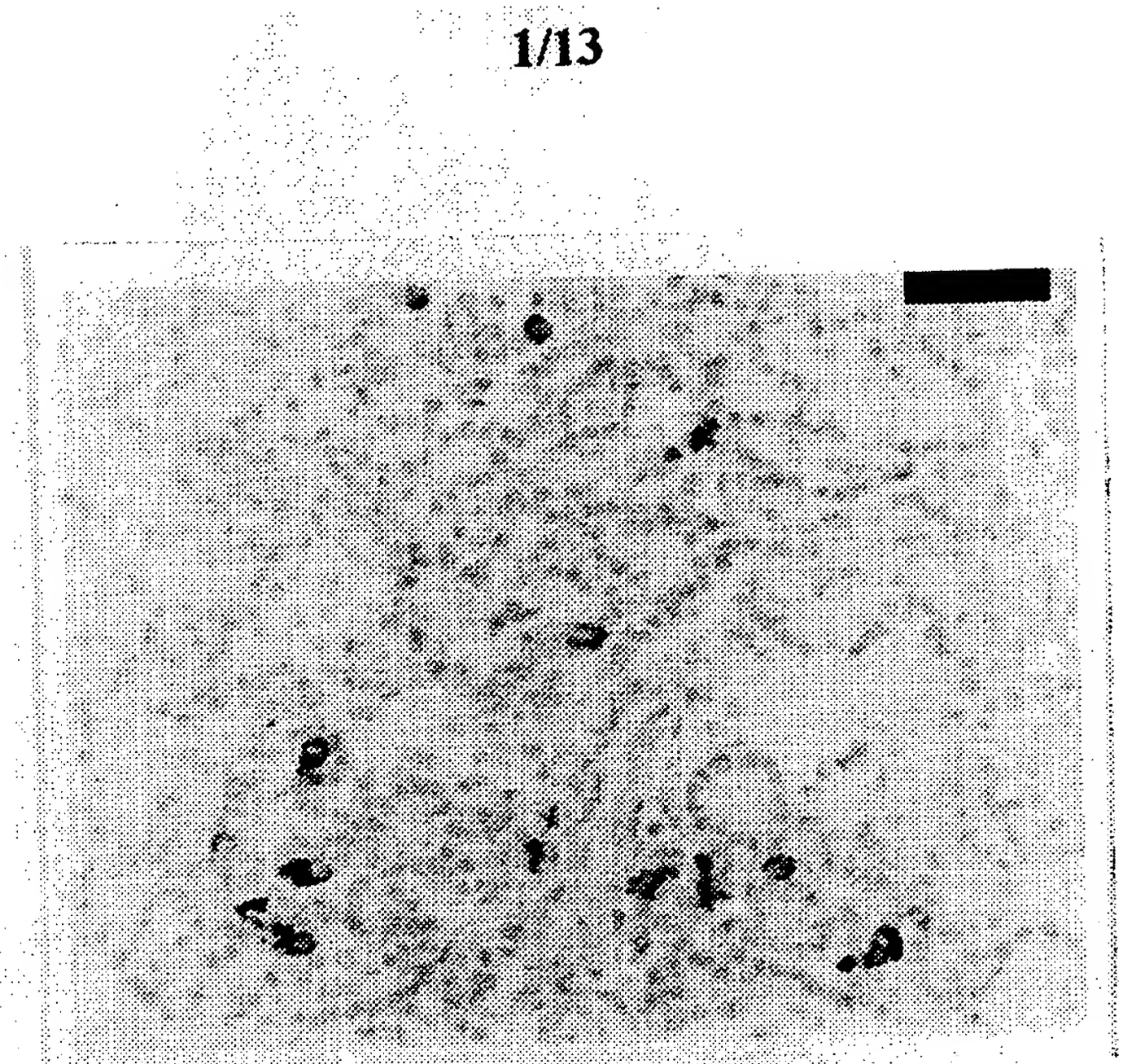


FIGURE 1A

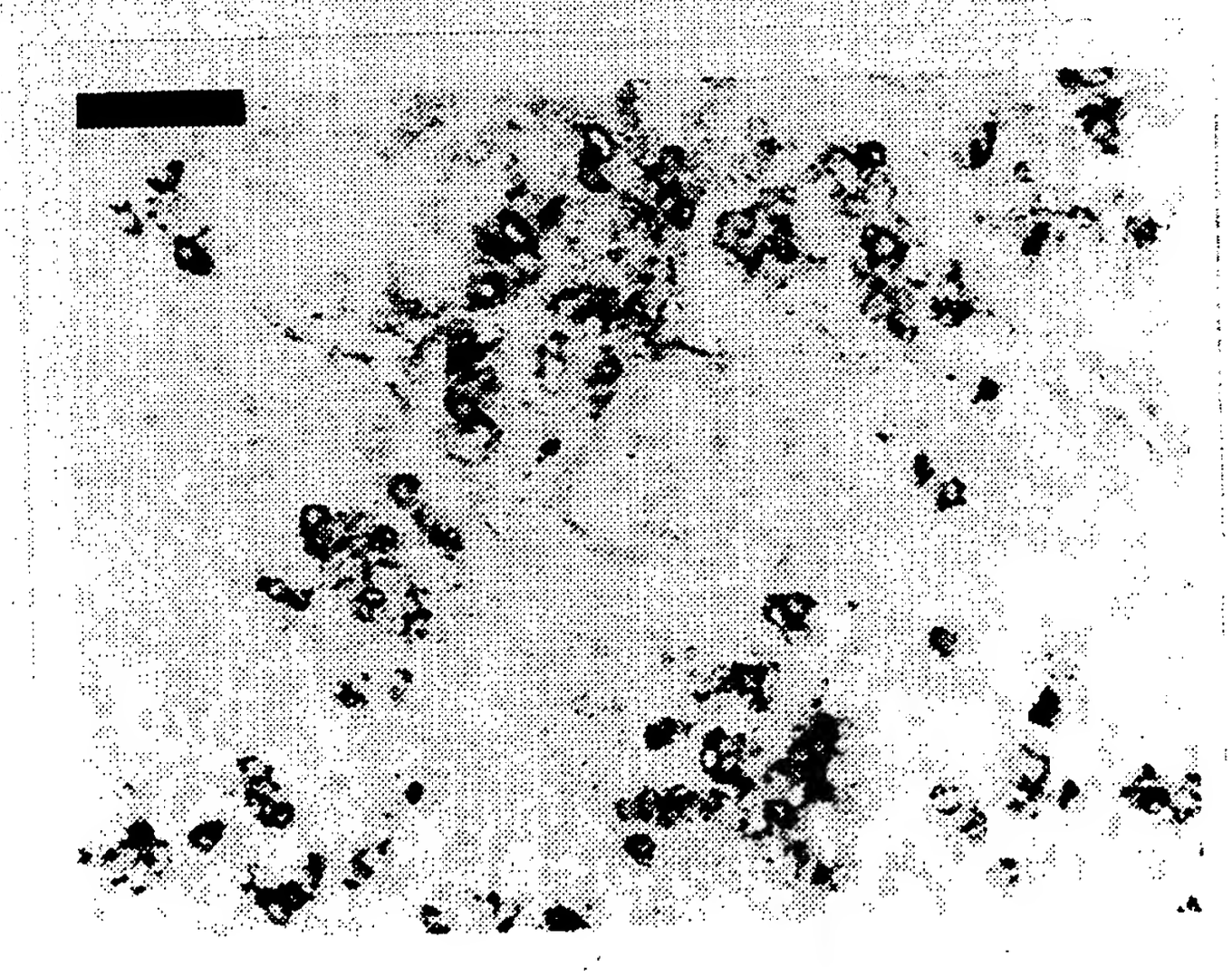


FIGURE 1B

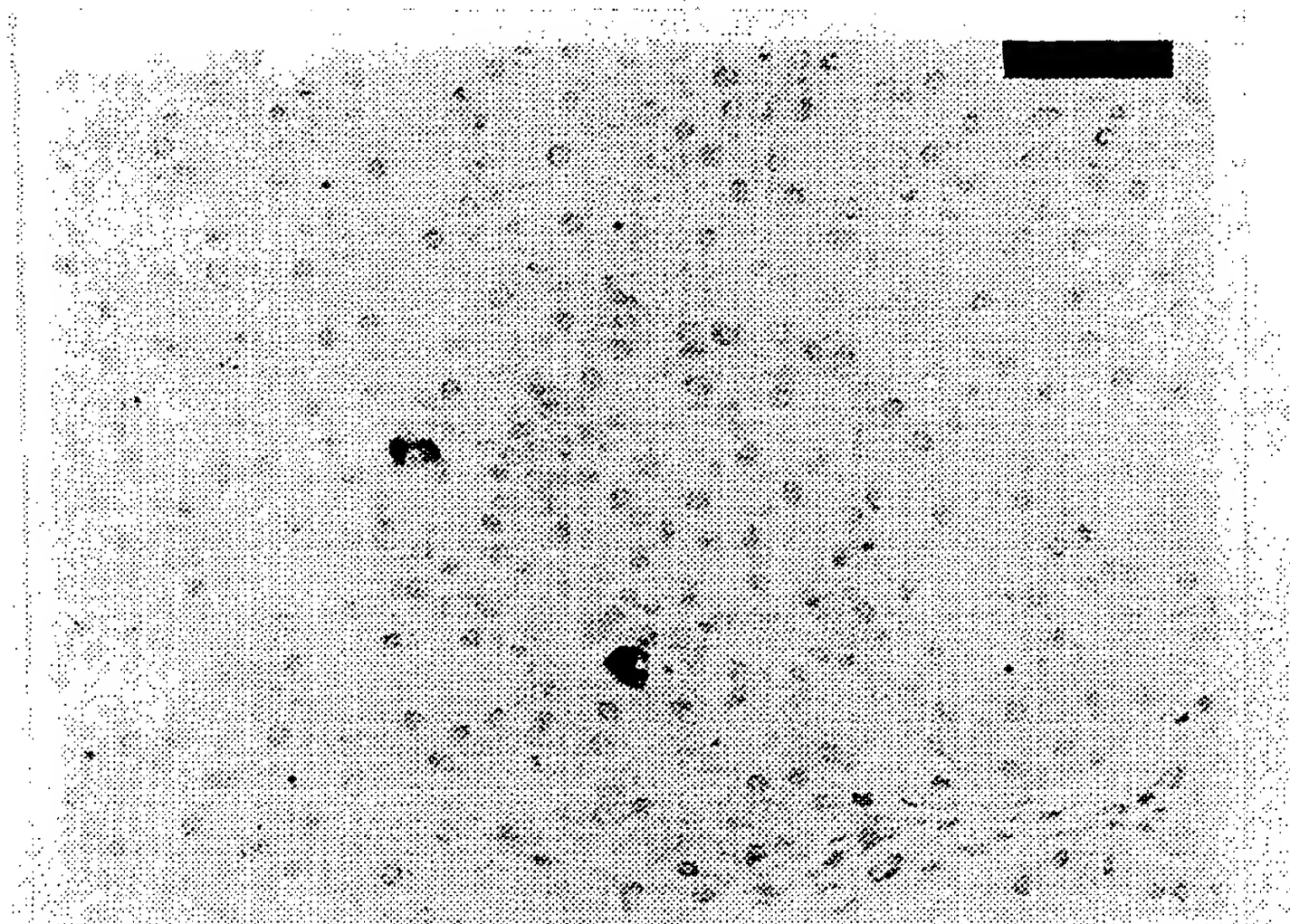


FIGURE 1C

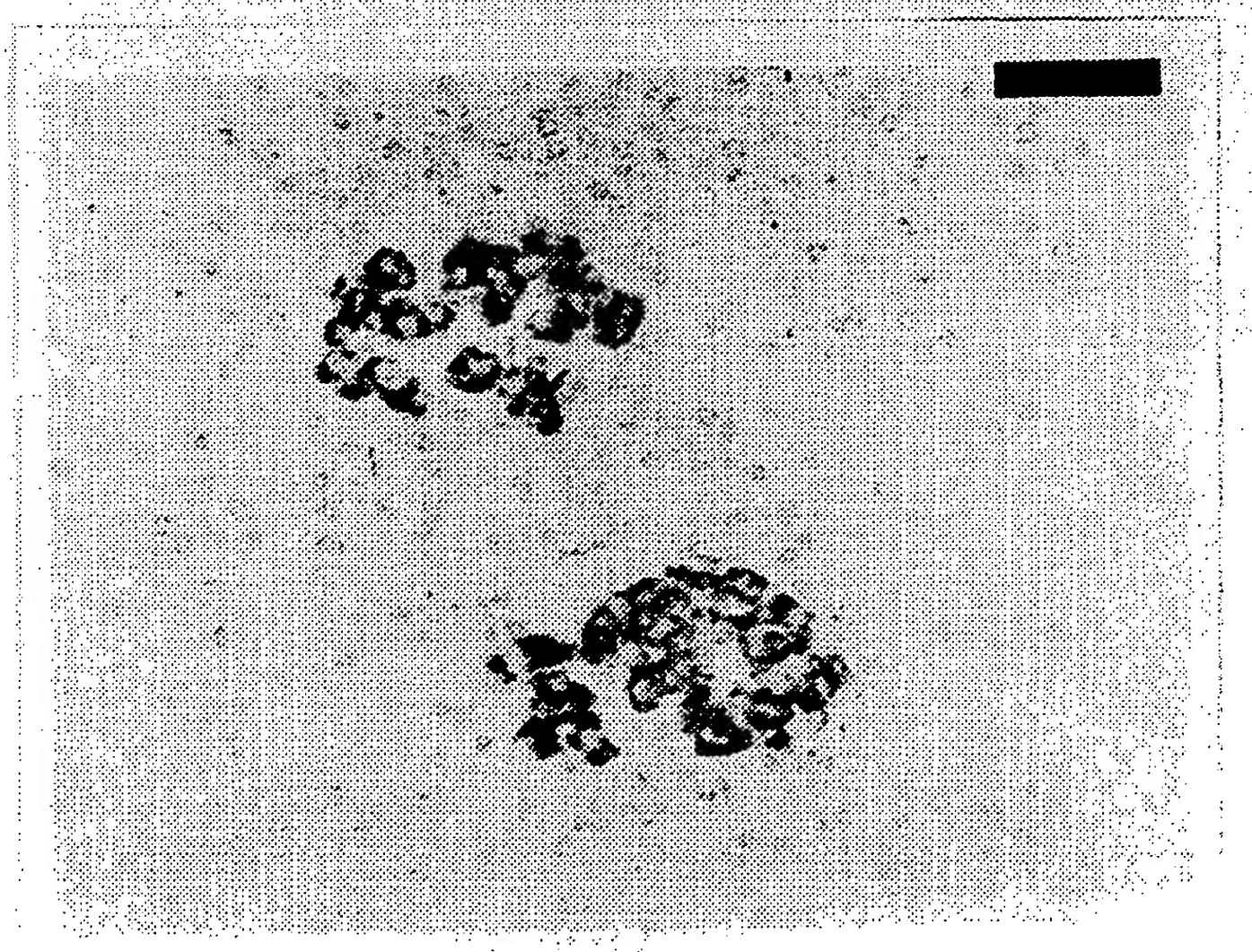
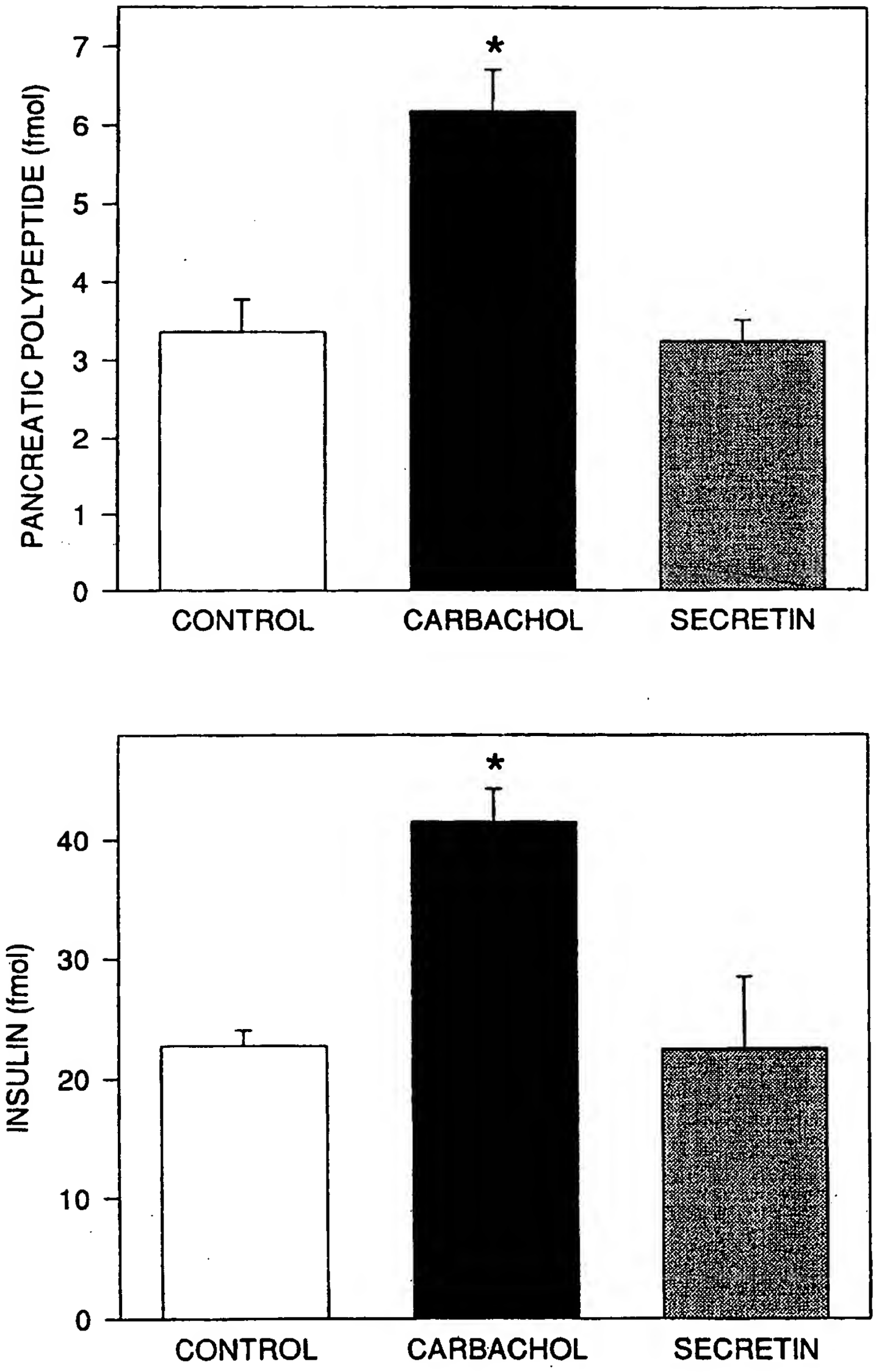


FIGURE 1D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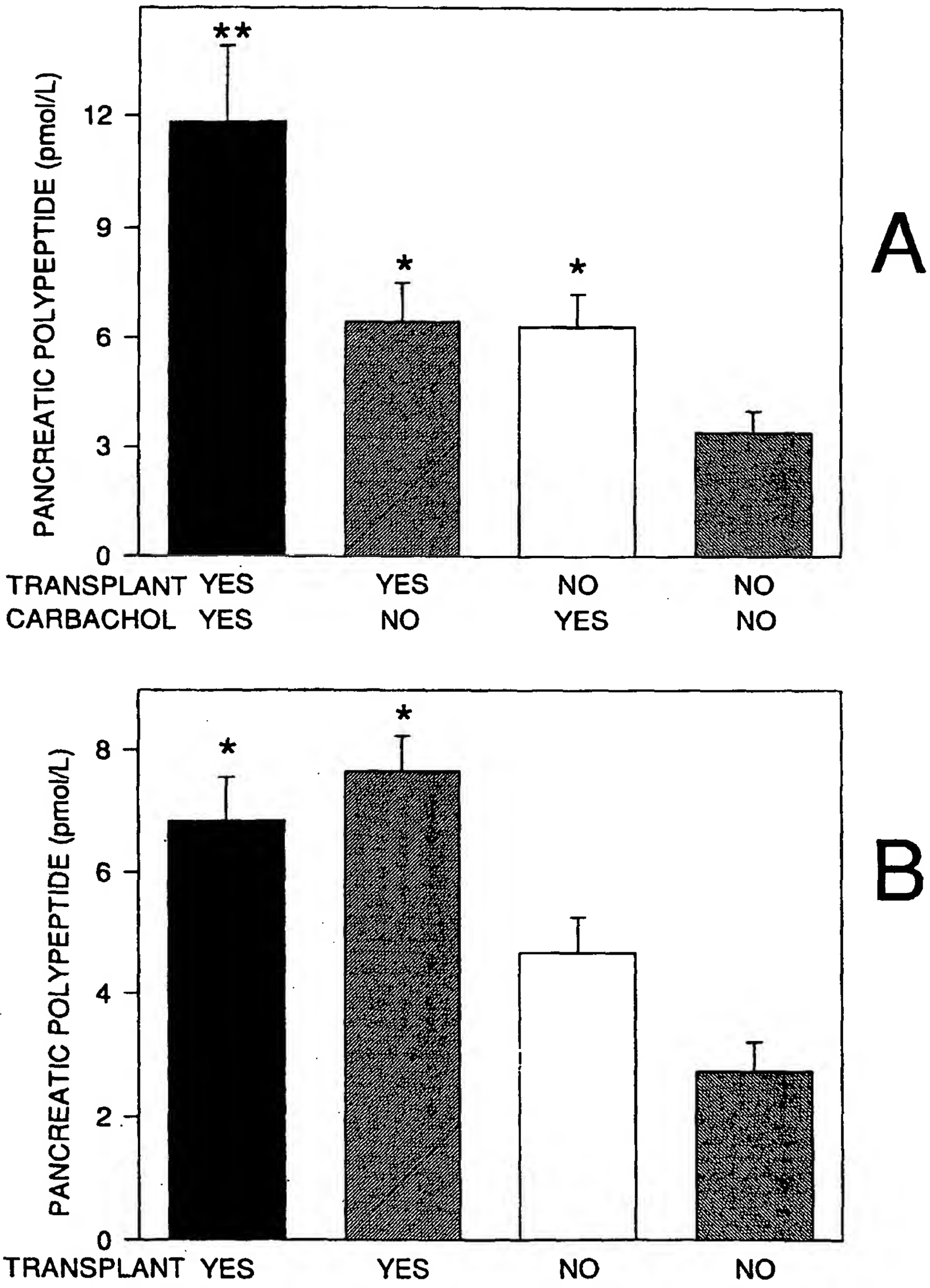
0
1
2
3
4
5
6
7
*
CONTROL
CARBACHOL
SECRETIN
INSULIN (fmol)
0
10
20
30
40
*
CONTROL
CARBACHOL
SECRETIN

FIGURE 2


A
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0
3
6
9
12
**
*
*
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CARBACHOL YES NO YES NO
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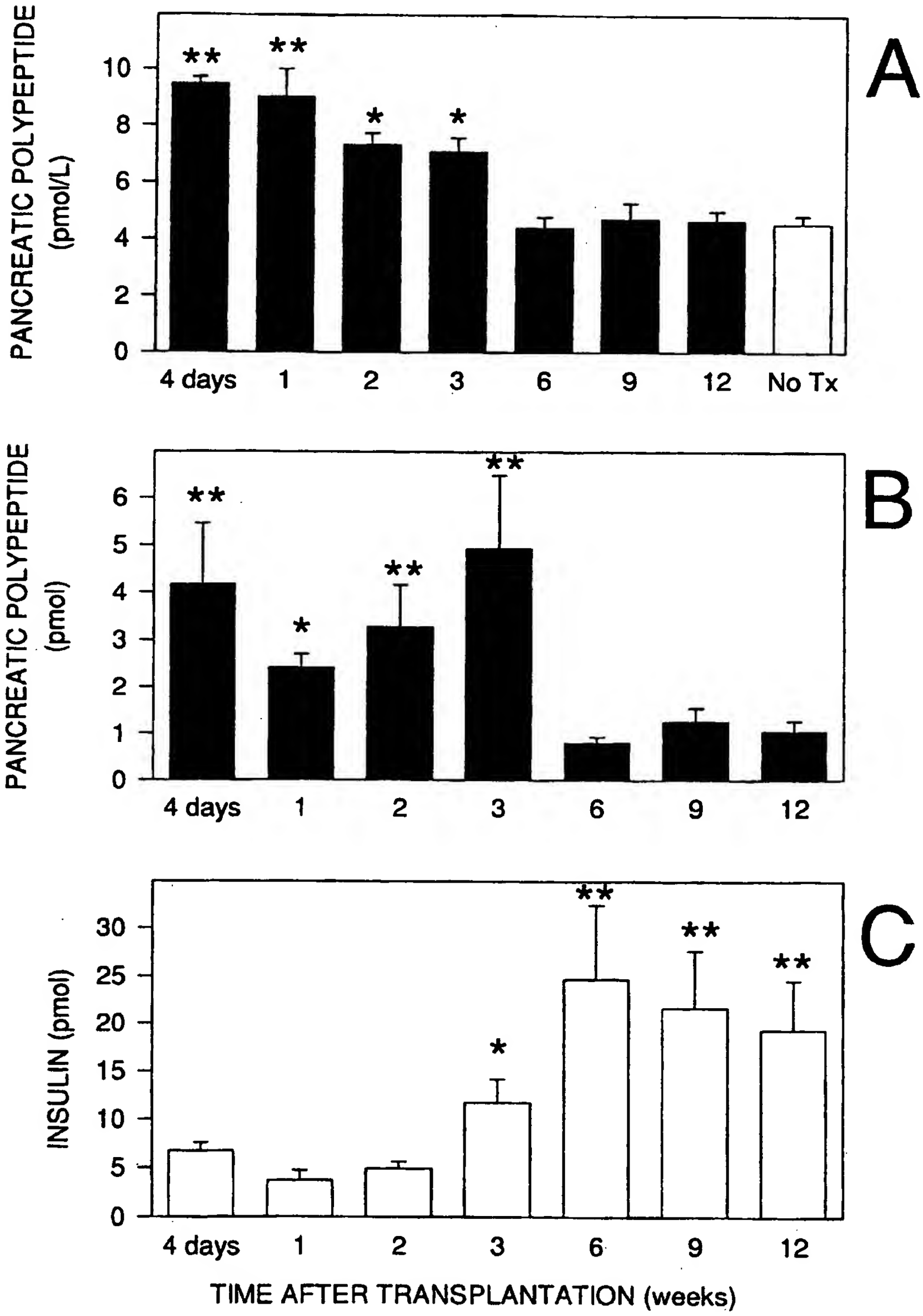
2
4
6
8
*
*
TRANSPLANT YES YES NO NO

FIGURE 3


A
PANCREATIC POLYPEPTIDE (pmol/L)
10
8
6
4
2
0
**
**
*
*
4 days
1
2
3
6
9
12
No Tx
B
PANCREATIC POLYPEPTIDE (pmol)
6
5
4
3
2
1
0
**
*
**
**
4 days
1
2
3
6
9
12
C
INSULIN (pmol)
30
25
20
15
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*
**
**
4 days
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2
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6
9
12
TIME AFTER TRANSPLANTATION (weeks)

FIGURE 4

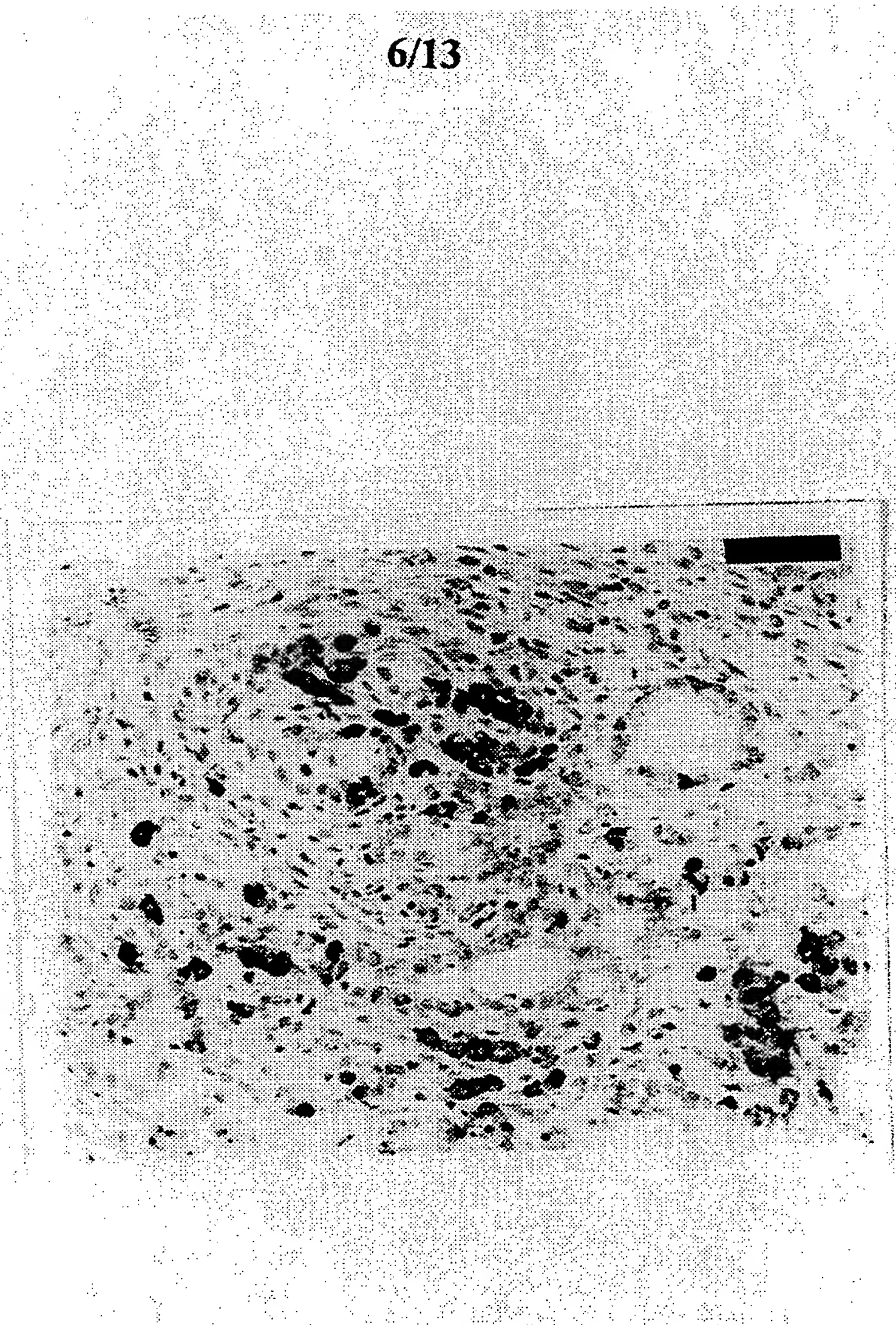
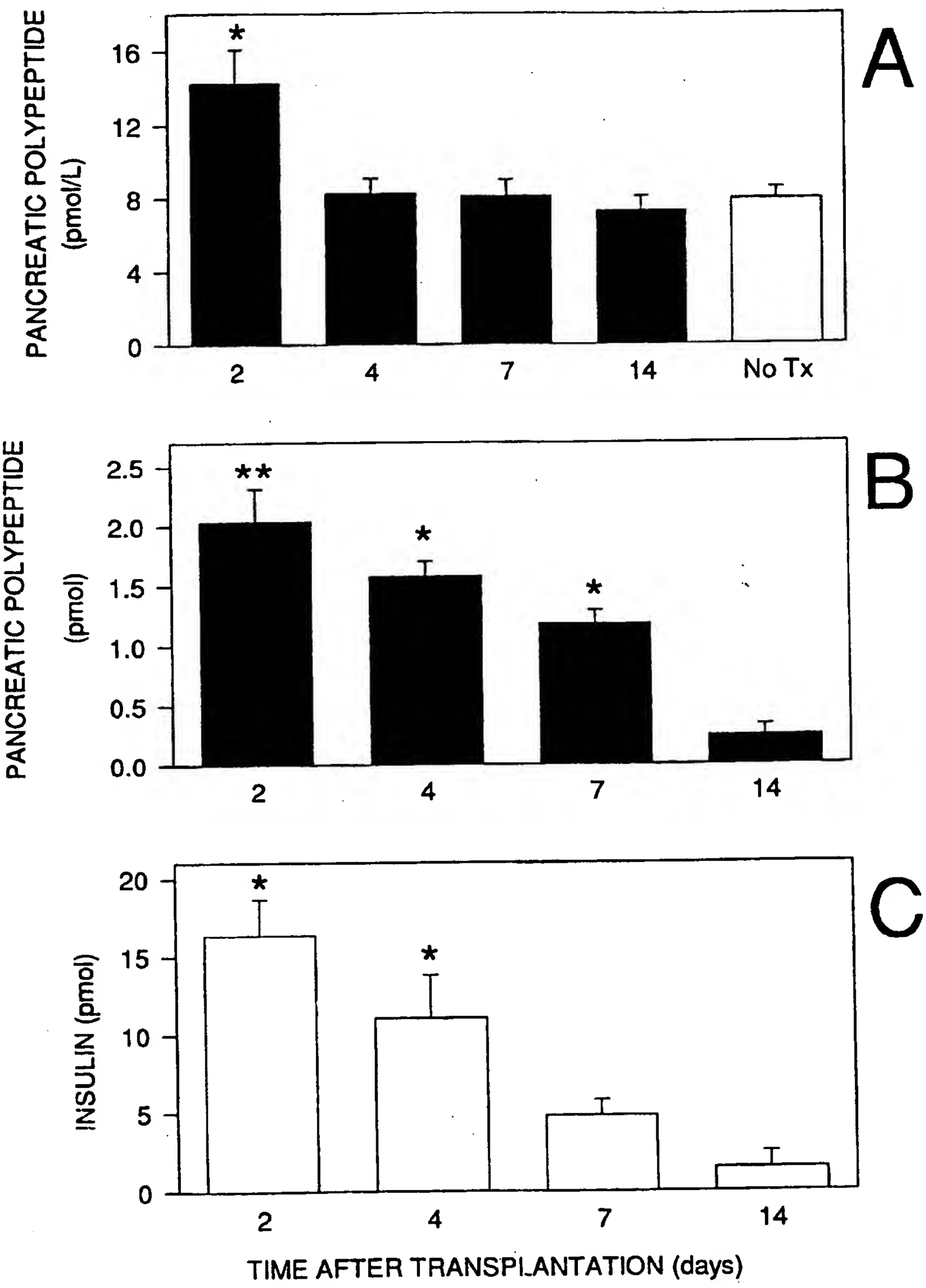


FIGURE 5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12
8
4
0
*
2
4
7
14
No Tx
B
PANCREATIC POLYPEPTIDE (pmol)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
*
2
4
7
14
C
INSULIN (pmol)
20
15
10
5
0
*
*
2
4
7
14
TIME AFTER TRANSPLANTATION (days)

FIGURE 6

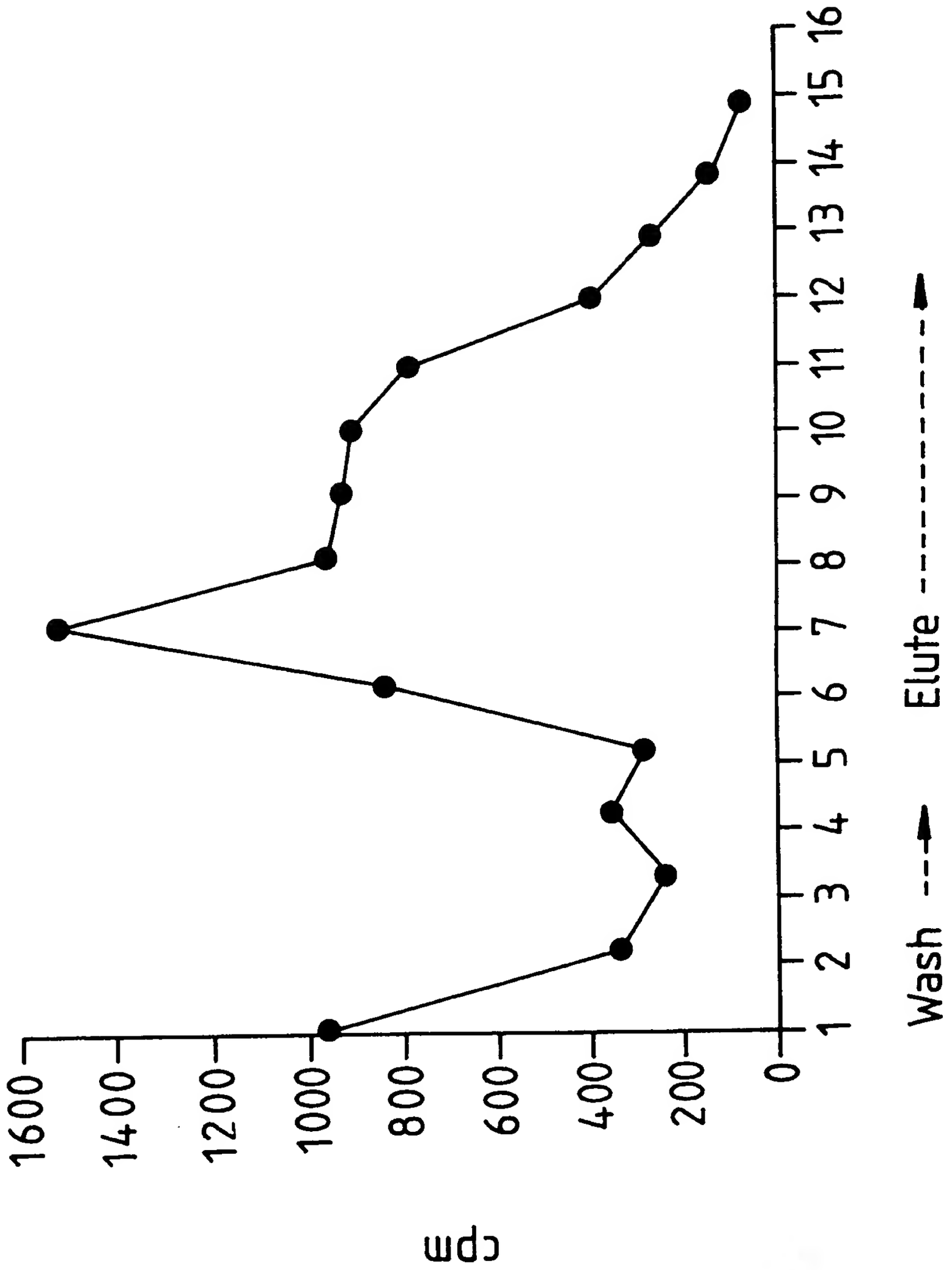
PIG PANCREATIC POLYPEPTIDE (Mol. wt. 4197)

ALA-PRO-LEU-GLU-PRO-VAL-TYR-PRO-GLY-ASP-*ASP*-ALA-THR-PRO-GLU-GLN-MET-ALA-GLN-TYR-ALA-ALA-*GLU*-LEU-ARG-ARG-TYR-ILE-ASN-MET-LEU-THR-ARG-PRO-ARG-TYR

HUMAN PANCREATIC POLYPEPTIDE (Mol. wt. 4182)

ALA-PRO-LEU-GLU-PRO-VAL-TYR-PRO-GLY-ASP-*ASN*-ALA-THR-PRO-GLU-GLN-MET-ALA-GLN-TYR-ALA-ALA-*ASP*-LEU-ARG-ARG-TYR-ILE-ASN-MET-LEU-THR-ARG-PRO-ARG-TYR

FIGURE 7


cpm
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1400
1200
1000
800
600
400
200
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
Wash ---→
Elute ----------→

FIG 8

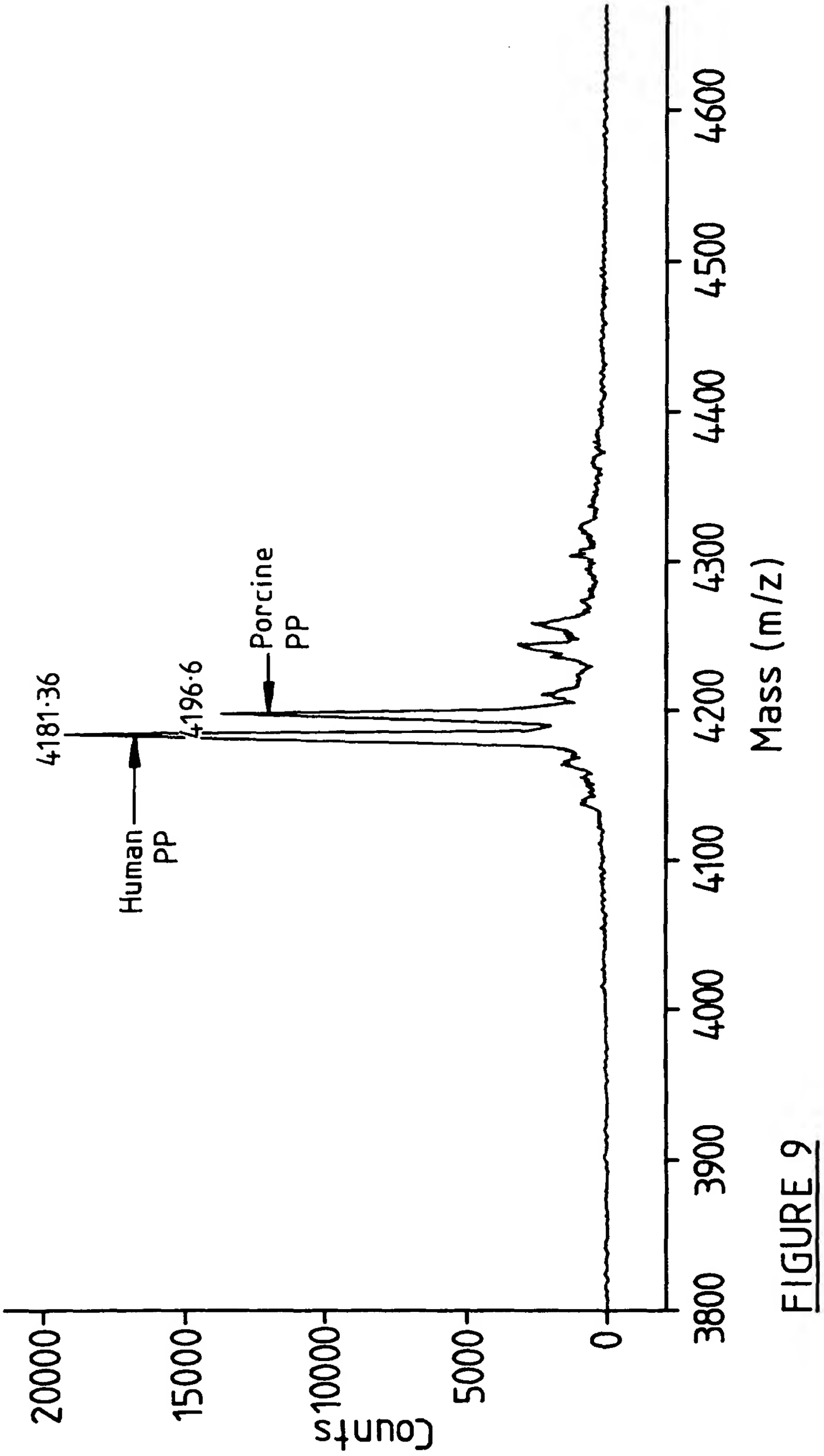
4181·36
4196·6
Human PP
Porcine PP
Counts
20000
15000
10000
5000
0
3800
3900
4000
4100
4200
4300
4400
4500
4600
Mass (m/z)

FIGURE 9

PORCINE ICOSAPEPTIDE (19 amino acids)

ASP-GLU-GLU-ASP-LEU-LEU-ASP-LEU-LYS-CYS-SER-SER-LEU-HIS-ALA-ALA-ALA-PRO-ARG

HUMAN ICOSAPEPTIDE (20 amino acids)

HIS-LYS-GLU-ASP-THR-LEU-ALA-PHE-SER-GLU-TRP-GLY-SER-PRO-HIS-ALA-ALA-VAL-PRO-ALA

FIGURE 10

PORCINE AMYLIN (32 amino acids)

ASN-MET-ALA-THR-CYS-ALA-THR-GLN-HIS-LEU-ALA-ASN-
PHE-LEU-ASP-ARG-SER-ARG-ASN-ASN-LEU-GLY-THR-ILE-
PHE-SER-PRO-THR-LYS-VAL-GLY-SER

HUMAN AMYLIN (37 amino acids)

LYS-CYS-ASN-THR-ALA-THR-CYS-ALA-THR-GLN-ARG-LEU-
ALA-ASN-PHE-LEU-VAL-HIS-SER-SER-ASN-ASN-PHE-GLY-
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FIGURE 11

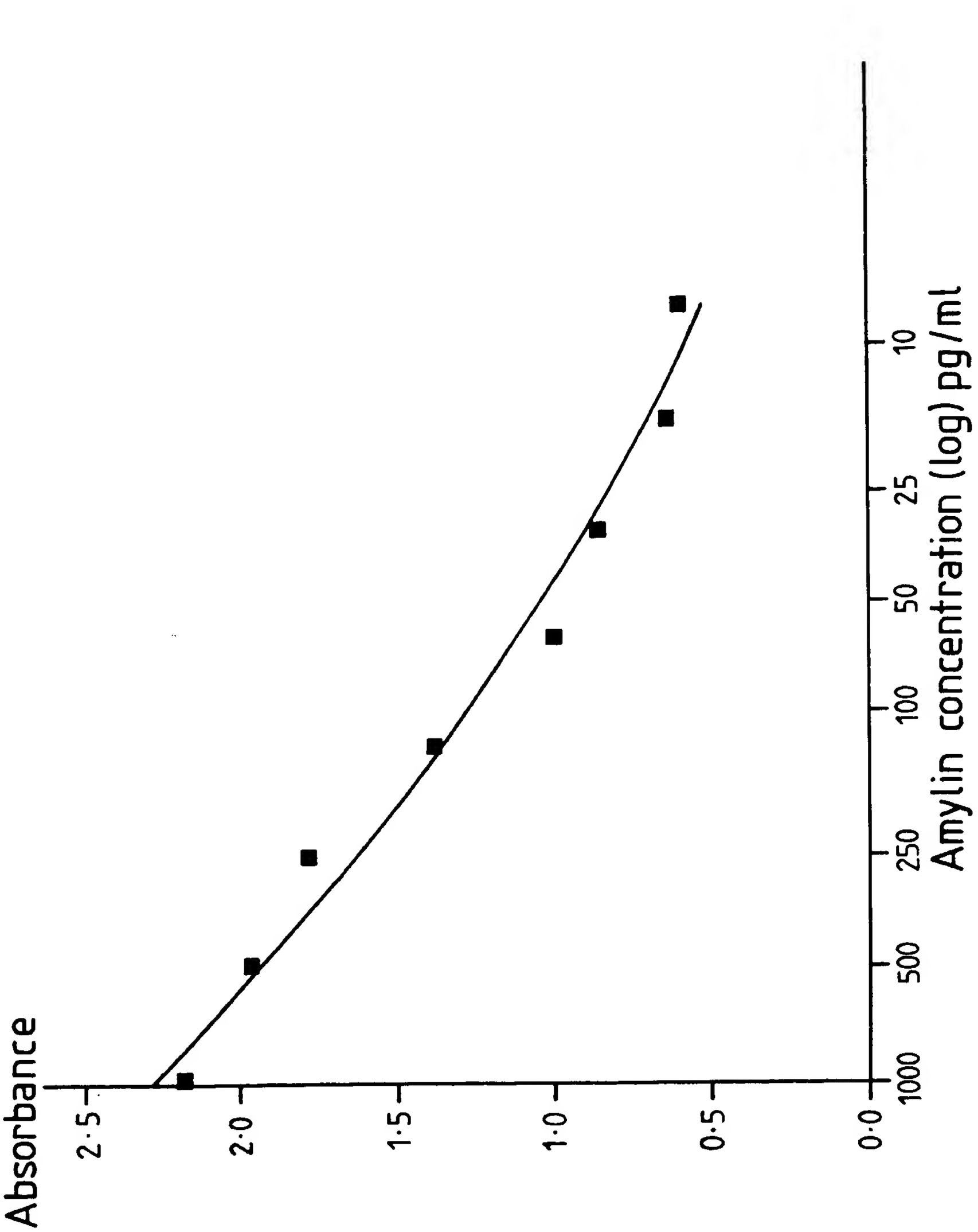
Absorbance
2·5
2·0
1·5
1·0
0·5
0·0
1000
500
250
100
50
25
10
Amylin concentration (log) pg/ml

FIG 12

SEQUENCE LISTING

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UNISEARCH LTD.

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<140>

<141>

<150> PP3490

<151> 1998-05-13

<160> 6

<170> PatentIn Ver. 2.0

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<211> 36

<212> PRT

<213> mammalian

<400> 1

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1 5 10 15

Met Ala Gln Tyr Ala Ala Glu Leu Arg Arg Tyr Ile Asn Met Leu Thr
20 25 30

Arg Pro Arg Tyr
35

<210> 2

<211> 36

<212> PRT

<213> mammalian

<400> 2

Ala Pro Leu Glu Pro Val Tyr Pro Gly Asp Asn Ala Thr Pro Glu Gln
1 5 10 15

Met Ala Gln Tyr Ala Ala Asp Leu Arg Arg Tyr Ile Asn Met Leu Thr
20 25 30

Arg Pro Arg Tyr
35

<210> 3

<211> 19

<212> PRT

<213> mammalian

<400> 3

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1 5 10 15

Ala Pro Arg

<210> 4

<211> 20

<212> PRT

<213> mammalian

<400> 4

His Lys Glu Asp Thr Leu Ala Phe Ser Glu Trp Gly Ser Pro His Ala
1 5 10 15

Ala Val Pro Ala
20

<210> 5

<211> 32

<212> PRT

<213> mammalian

<400> 5

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1 5 10 15

Ser Arg Asn Asn Leu Gly Thr Ile Phe Ser Pro Thr Lys Val Gly Ser
20 25 30

<210> 6

<211> 37

<212> PRT

<213> mammalian

<400> 6

Lys Cys Asn Thr Ala Thr Cys Ala Thr Gln Arg Leu Ala Asn Phe Leu
1 5 10 15

Val His Ser Ser Asn Asn Phe Gly Ala Ile Leu Ser Ser Thr Asn Val
20 25 30

Gly Ser Asn Thr Tyr
35

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00361

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: G01N 33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC A61K 049, 051, C12Q, G01N 033

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See extra sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93/03381 A, (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM), 18 February 1993 - claims 1-5	1
X	WO 93/23435 A, (AMYLIN PHARMACEUTICALS, INC.), 25 November 1993 - page 6, line 26 - page 7, line 2.	20
X	WO 95/18609 A, (CITY OF HOPE), 13 July 1995 - page 74, line 26 - page 75, line 10	1

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 July 1999	- 2 AUG 1999
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer **ISOBEL TYSON** Telephone No.: (02) 6283 2563

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00361

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97/35600, A (AMYLIN PHARMACEUTICALS, INC.), 2 October 1997 - entire document	1, 6
X	Scand J Clin Lab Invest, 1997; 57: 695-702, BRIMNES DAMHOLT, M. et al - especially page 695, line 1 - page 696, line 14	1-3
X	Clin Transplantation, 1998; 12: 56-64, ELMER, D.S. et al - especially page 62-63	1
X	J Surgical Research, 1991; 50: 24-29, ZHENG, T. et al - especially pages 27-28	1, 2
A	Scand J Gastroenterology, 1977; 12: 923-927, HALLGREN, R. et al - entire document	1-3
A	Acta Endocrinologica, 1984; 105: 72-77, JONUNG, M. et al - entire document	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 99/00361

Box B cont'd.

Electronic databases used:

WPAT, JAPIO: icosapeptide, pancreatic peptide, amylinfetal islet like cell cluster, transplant
MEDLINE: pancrea?, transplantion, pancreatic polypeptide, icosapeptide, amylin
CHEMICAL ABSTRACTS: pancrea?, transplantion, pancreatic polypeptide, icosapeptide, amylin

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU 99/00361

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
WO	93/03381	AU	23479/92	US	5340721	US	5484707
WO	93/23435	AU	43779/93	CA	2113278	EP	594843
		JP	7508173	NZ	253449		
WO	95/18609	AU	674339	CA	2077461	EP	621895
		EP	696193	JP	7500254	WO	9405777
		CA	2157017	AU	70906/94		
WO	97/35600	AU	27216/97				
							END OF ANNEX